HENRY FORD HOSPITAL

International Symposium

Mechanisms of Hypersensitivity

The symposium was sponsored by the Henry Ford Hospital Detroit Michigan and held at the hospital March 27, 28, 29 1958

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HENRY FORD HOSPITAL INTERNATIONAL SYMPOSIUM

Mechanisms of Hypersensitivity

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FIRST EDITION

Previously Published Henry Ford Hospital Symposia

1	The Dynamics of Virus and Rickettsial Infections	October 1953
2	The Hypophyseal Growth Hormone, Nature and Actions	October 1954
3	Cardiovascular Surgery: Recent Studies in Physiology, Diagnosis and Techniques	March 1955
4	Enzymes: Units of Biological Structure and Function	November 1955
5	The Leukemias: Etiology, Pathophysiology and Treatment	March 1956
6	Hepatitis Frontiers	October 1956
7	Reticular Formation of the Brain	March 1957

Published in Great Britain
by J. & A. Churchill Ltd., London

PRINTED IN THE UNITED STATES OF AMERICA

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Foreword

For over fifty years Hypersensitivity has been recognized as an intriguing phenomenon with far reaching manifestations In this rapidly advancing era of medicine and with the accelerated use of an ever growing list of chemicals biologicals antibiotics and newer surgical techniques which involve tissue transplants it is not surprising that untoward tissue responses or altered reactions commonly accepted as allergic in nature are encountered with increasing frequency There is a growing feeling that some of the newly recognized disease entities seen in clinical medicine may well be manifestations of Hypersensitivity

Although certain aspects of Hypersensitivity have been explored in recent meetings held in the United States and abroad it was the opinion of the Henry Ford Hospital Symposium Committee that a symposium devoted entirely to the study of the fundamental processes involved merited serious consideration

On the basis of this decision the Committee with expert guidance and assistance from a National Advisory Committee set about the task of formulating a program which would include the important features of this subject Material submitted for possible inclusion in such a program was voluminous and covered so many varied aspects of the subject that it was necessary to channel subject material toward the basic mechanisms involved By necessity it was possible to select only a few of many eminently qualified scientists for participation in the formal program

Thus for the first time there was the opportunity for scientists of various disciplines dedicated to a common interest to present their most recent and hitherto unpublished research data and to challenge or substantiate the basic concepts of hypersensitivity Sixty five formal participants and 450 investigators from all parts of the world were on hand to discuss research techniques and results

We trust that those individuals who attended the three day session and those who read this published volume of the proceedings will be stimulated to continue their research with renewed vigor ever applying newer methods and newer knowledge which will lead to a better understanding of the fundamental Mechanisms of Hypersensitivity

JOSEPH H SHAFFER M D
Chairman

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I

Heterogeneity of Antibodies

Chairman MERRILL W CHASE PH D (*New York New York*)

Chairman's Remarks

Our voluntary presence here filling this room can be viewed only as a general recognition of the renewed interest and ever expanding data obtained by experimental means in a subject that has intrigued man for as long as he has known of the abnormal reactions that certain individuals experience in environments and in circumstances that have been indifferent for others

Having reached a decision that the field of Hypersensitivity was a subject of proper interest for an International Symposium the Committee of the Henry Ford Hospital cast around for an Advisory Committee to assist them in the planning The results of our meetings imperfect as they are are before you on the program It was our decision primarily to explore certain areas rather than to invite a roster of well known persons to present whatever work they were currently engaged upon In this way we hoped to provide a roundness that would serve to fix special areas of inquiry and (in a degree) subordinate the so called last word to a better appreciation of the general areas of investigation Questions always arise as to the choice of invited speakers I am sure that hardly one of you here would have made our precise selection of subjects or speakers But diversity in points of view has been represented within the Advisory Committee I judge that the area of our agreement will have provided you with something that will offer food for thought suggest some new experiments to each of you who are in experimental laboratory science and perhaps produce a new understanding of the interrelationships of the various parts of the presently known subject of Hypersensitivity

The program is crowded obviously and must be adhered to rigidly if justice is to be done to all persons on it The Advisory Committee has been in agreement that the use of slides in general discussion is often overcostly of the time of an audience Every slide means a new orientation for the hearer how the slide is constructed what the basis was for the data drawn upon and what the interpretation and balance are between the various elements represented on the slide Old hands at conferences recognize that contributions of discussors are of very real value but feel that what the

discussor has to offer in a limited time is often more appropriately put in words than in a presentation of actual laboratory data and secondarily from them an interpretation of the data There is no intent to cut down the making of real contributions But the contributions can we feel be offered and accepted by an audience better in words than through the medium of detailed evidence In instances where data offered by a person in the audience are of such critical importance that they merit being presented in the volume that may well follow this symposium space will I am sure be provided for those whose contributions have shown themselves to be most germane

There is little doubt that discussion will be rife even if confined to small groupings of individuals over the entire three days

It is the hope of the Henry Ford Hospital and of the Local Committee and of the Advisory Members that you will find the three days spent together rewarding and stimulating We shall have succeeded if you carry away at the conclusion of the symposium something that is of personal value to you

I

*Qualitative Differences in Antibodies**

DAVID W TALMAGE MD
(Chicago Illinois)

It is appropriate to open a discussion of the diversity of antibodies by defining the limits within which all antibodies may be unified It is probable that general agreement can be obtained for the statements that (1) all antibodies are relatively large proteins ( ) the production of antibodies is accelerated by the injection of antigens and (3) the antibodies so produced have a relatively specific affinity for the antigen injected [24] A fourth and more controversial statement is that antibodies cannot be divided into broad general classes on the basis of their reactions with antigens

It was the recognition of the broad indivisibility of antibodies contained in the last statement that led Zinsser [25] to formulate the Unitarian Theory It seemed unreasonable to Zinsser to divide antibodies into agglutinating precipitating and hemolytic types because it could be demonstrated that the same antibody could perform in all three reactions This principle is probably true today but the situation is more complex and needs restating It is the thesis of this paper that antibodies are indivisible not because they are so much alike but paradoxically because they are so diverse The manifestations of antigen antibody reactions depend on so many factors which vary independently that it is impossible to assign an all important role to any one factor The injection of a single antigen results in the formation of such a wide spectrum of antibodies with crossing patterns of reactivity that no reasonable division into types is possible While one speaks of precipitating antibodies and nonprecipitating antibodies it is difficult to define precisely what is meant by these terms and it is possible that no sharp line exists between them Strictly speaking it would be more correct to refer to a "precipitating antigen antibody system than to a precipitating antibody

The difficulties in classifying antibodies may be conveniently illustrated with the hemolytic system Using electrophoresis on a starch block [26] it was possible to separate a rabbit antiserum to sheep cells into two peaks of antibody activity (Figure 1) One of these peaks showed a high level of

* This work was done with the support of Grant E 1378 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health U.S Public Health Service

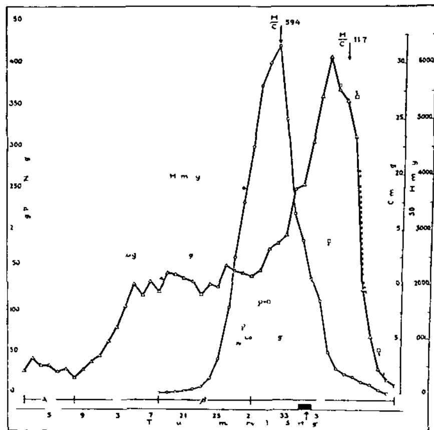


FIGURE 1 Electrophoretic distribution of the anti Forssman hemolytic and combining activities of a globulin fraction prepared by ammonium sulfate precipitation The serum was obtained from a splenectomized rabbit given 8 intravenous injections of 1 ml 10 per cent heated stromata per kilogram

hemolytic activity the other was demonstrated by its ability to block the absorption of labeled antibody Our initial inclination to consider the first peak as hemolytic antibody and the second nonhemolytic was frustrated by the finding that the second peak possessed a lower but separate and distinct hemolytic activity (Figure) It is possible that all antibodies are potentially capable of eliciting all the various antigen antibody reactions but with different efficiency and threshold If this is true it is impossible to divide antibodies into sharply defined types such as complete and in complete or allergic and nonallergic types While the various labels are convenient to use they are dangerous if they serve to cover our ignorance of the mechanisms by which they act

In general antigen antibody reactions depend on several independent primary properties of antibody and on factors not involving antibody

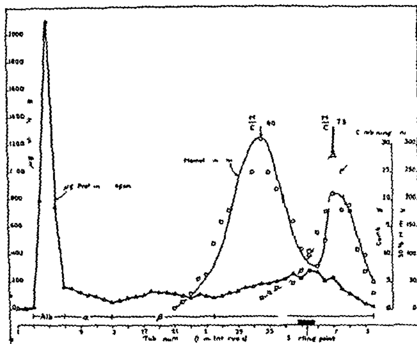


FIGURE 2 Electrophoretic distribution of the anti Forssman hemolytic and combining activities of an antiserum to sheep red cells from a rabbit immunized by repeated injections over a period of 4 months

The primary properties of antibodies may be listed as follows (1) size ( ) charge (3) composition (4) amino acid sequences (5) gross configuration and (6) configuration of combining site In general antibodies vary in each of these primary properties with little or no correlation between them It is difficult to attach causal significance to a few general relationships which may be noted For example antibodies which are found by centrifugal separation in the slowly sedimenting fractions of rabbit globulin are generally found in the slowly migrating gamma globulins on electrophoresis Rabbit antibodies and gamma globulins studied to date have been found to be identical with regard to amino acid composition and the sequence of the five terminal amino acids[18 21 21] In contrast to rabbit globulins bovine and human gamma-globulins were found by Press and Porter to have several different N terminal amino acids No correlation could be found between the N terminal amino acid and the rate of flow through a starch chromatographic column except in the case of alanine which was found as a terminal acid only in the rapidly flowing fractions Little is known about the gross configuration of antibody molecules except that studies which have been made are compatible

with an elongated shape[1] The configuration of the entire surface of the molecule including the part complementary to antigen may play a role in the flow of the molecule through starch columns or blocks and perhaps is one of the important factors determining the slow diffusion of 'reagins from the site of injection[11] or the varied distribution of antibody molecules in the body compartments[7]

The configuration of that portion of the antibody which combines with antigen determines the specificity of the antibody and its affinity for antigen Specificity has several different levels of precision With complex cellular antigens there is diversity with respect to the various molecular entities in the complex antigen With a highly purified protein antigen the possibility exists of antibodies complementary to different areas on the surface of the same molecule This seems especially likely because the combining sites on the antigen and antibody molecules are relatively small The demonstration by Eisen Carsten and Belman[8] of three specificities of antibody to the same azoprotein indicates that such variation in site of attachment does occur

If it is desired to study the diversity of antibody specificity with respect to a single antigenic determinant it is necessary to turn to simple chemicals which become antigenic when attached to proteins Antisera prepared against such antigens are as monospecific as we can hope to get However even in these systems diversity of antibody is demonstrated in two entirely separate dimensions (1) by their ability to cross react with related antigens and (2) by their affinity for the homologous hapten An example of the first type of heterogeneity was demonstrated more than twenty years ago by Landsteiner and van der Scheer[13] Antisera prepared against a hapten horse serum antigen were tested for precipitin against the homologous hapten and two related haptens diazotized to chicken serum The results indicated that there were at least two types of antibodies in the serum one absorbed by one of the heterologous haptens attached to red cell stromata and the other absorbed by the other Only the homologous hapten absorbed all of the antibodies

The affinity of haptens for antisera was first measured by Marrack[17] using equilibrium dialysis and by Landsteiner and van der Scheer[12] using hapten inhibition of precipitation The first technique has been used with considerable success by Lerman[14,15] and Eisen Carsten and Karush[4,6] The reports to date have uniformly indicated that heterogeneity of affinity exists for a single chemical determinant I will leave the further discussion of this problem to Dr Karush

A partial list of antigen antibody reactions includes (1) aggregating reactions (2) hemolysis (3) toxin neutralization (4) complement fixation (5) protection from living organisms (6) Arthus reactions (7) anaphylactic shock in the intact animal (8) muscle contraction (Schultz

Dale) and (9) wheal and erythema. Each one of these reactions is enormously complex with a variable threshold and a whole spectrum of reactions. To illustrate this the first two of these reactions will be discussed.

Several relatively comprehensive theories of antibody antigen precipitation have been presented based on a lattice formation of antigens and antibody molecules. The most unequivocal evidence for the importance of lattice formation was the finding of Pauling, Pressman and Campbell[6] that a compound hapten containing one each of two determinant groups precipitated with a mixture of the antisera against the two determinants but not with either antiserum separately. Perhaps the most rigorous mathematical analysis of the complex series of reactions preceding precipitation was made recently by Goldberg.

In his analysis Goldberg made the following assumptions (1) The antigen and antiserum mixture consisted of known concentrations of bivalent and univalent antibody molecules and antigen molecules of known valence a given fraction of which had reacted. ( ) All antigen and antibody valences reacted independently regardless of the size or shape of the complexes in which they occurred. (3) No cyclic complexes were formed. From this model a formula was derived which gave the most probable distribution of complexes of any size or composition.

It was also possible to derive a much simpler formula which gave the critical conditions at which the material passes into the form of very large aggregates. For an antiserum containing only bivalent antibody this was $rp^2 = \frac{1}{V-1}$ where r is the ratio of antigen to antibody sites, p is the critical fraction of antigen sites bound and V is the valence of antigen. Figure 3 gives a graphical representation of this formula for bivalent antibody and for antigen valences of 6, 100 and 10 000 (solid horizontal lines). For an antigen valence of the formula predicts that aggregation can occur only in a mixture of equal numbers of antigen and antibody combining sites. With an antigen valence of 6 the formula predicts that aggregation can occur over a twenty five fold range of antigen to antibody ratios and for an antigen valence of 100 over a ten thousand fold range of antigen to antibody ratios. This is compatible with the finding that the precipitation of small antigen molecules is sharply inhibited in antigen excess and may be inhibited in antibody excess.[20]

Although the vertical coordinate in Figure 3 is actually rp the plot is given in terms of the per cent of antibody sites bound rp, the per cent of antigen sites bound p, and the ratio of these sites to each other r. This is done in order to consider the situation which exists when a significant fraction of both antigen and antibody valences are free at equilibrium. This occurs whenever the concentration of antibody is low relative to

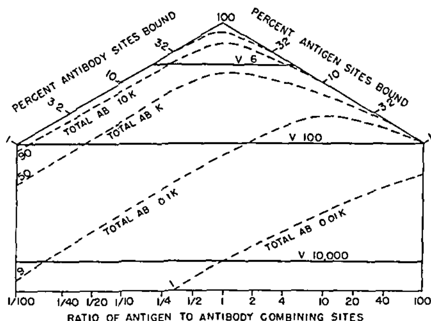


FIGURE 3 A diagrammatic representation of the limiting conditions for the formation of large aggregates as predicted by the Goldberg theory For a bivalent antibody of a given concentration relative to its equilibrium constant K and for an antigen of given valence V the conditions required for aggregation obtain when the appropriate broken curve is above the indicated solid horizontal line

its dissociation constant K_D If the assumptions of the Goldberg model are made the fraction of antigen valences reacting at equilibrium p can be readily calculated from the formula $p = \frac{S - \sqrt{S^2 - \frac{4}{r}}}{2}$ where $S = \frac{1 + R + r}{r}$ and R is the ratio of K_D to the concentration of antibody combining sites The broken lines in Figure 3 give the results of these calculations for total antibody concentrations equal to 100 K_D 10 K_D K_D *0 1* K_D *and 0 01* K_D *respectively This formulation would predict that for* an antigen valence of 6 the critical conditions could not be reached if the antibody concentration was equal to or less than K_D For larger valences of antigen aggregation may occur at lower concentrations of antibody

These predictions are consistent with the well known fact that agglutination (large valence of antigen) is a more sensitive test of antibody than precipitation and with the finding that with sera obtained early in the course of immunization dilution of the antiserum produces a marked effect on the amount of precipitation[30] An example of this dilution effect is shown in Figure 4 which plots the fraction of labeled antigen (I^{131}

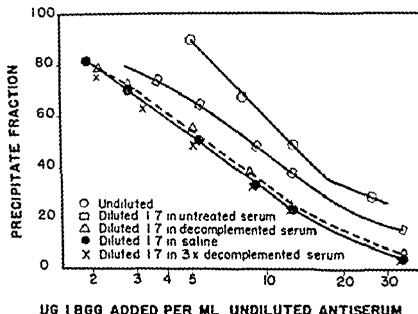


FIGURE 4 Antigen precipitate fraction curves from an undiluted antiserum obtained 10 days after injection of BGG in saline and from the same serum diluted in saline in untreated normal rabbit serum and in normal rabbit serum decomplemented once and three times with 50 μg /ml specific precipitate

bovine gamma globulin) precipitated against the amount of labeled anti gen added to a constant volume of antiserum The dilution of the antiserum by a factor of 7 resulted in a twofold decrease in the amount of antigen precipitated per milliliter of undiluted serum

We may summarize as follows the factors which determine whether a given mixture of antigen and antibody will precipitate if we consider only the assumptions of the Goldberg model (1) the valences of the antigen and antibody and ( ) the concentrations of the antigen and anti body relative to each other and to the dissociation constant of their reaction

Although this is an excellent basis for any consideration of the pre cipitin reaction there is evidence that additional factors are involved The nonagglutinating or blocking Rh antibodies have been shown to agglutinate Rh positive cells treated with enzyme or cells of an unusual type D [9] It is difficult to ascribe their lack of agglutinating capacity either to univalence or to lack of affinity for antigen Campbell Sturgeon and Vinograd [2] have shown that the agglutinating antibody in this system has a relatively high sedimentation constant (19 4S) and probably possesses a large molecular weight (approximately one million) The most reasonable interpretation of their data is that the distance between

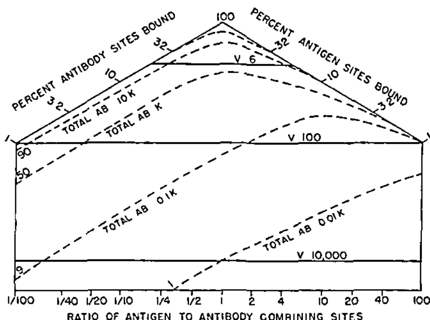


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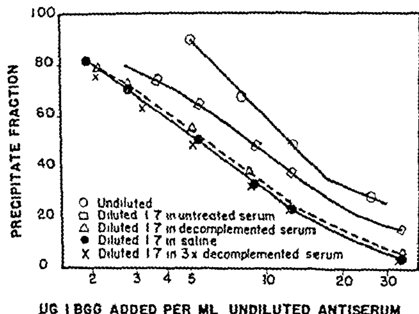


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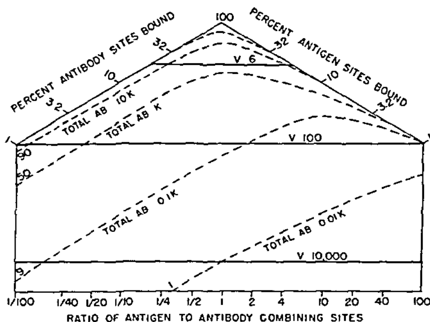


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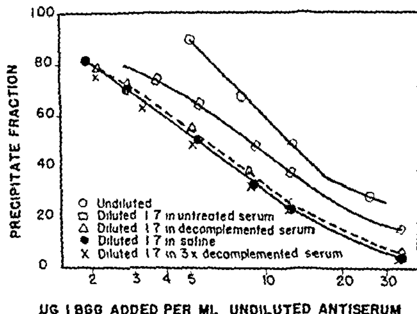


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the valences of the smaller molecule is too short to bridge the gap between the combining sites on two red cells This finding strongly suggests that in general the position of the valences on the antigen and antibody molecules as well as their number is important in determining whether aggregation will be the outcome of any particular mixture

The results presented in Figure 4 indicate that complement also plays an important role in precipitation The dilution of the antiserum in untreated normal rabbit serum resulted in a smaller dilution effect than dilution in decomplemented rabbit serum Weigle and Maurer [31] have recently given convincing evidence that complement can cause the partial precipitation of soluble antigen antibody complexes in far antigen excess

A third factor which complicates the Goldberg model is the probable formation of cyclic complexes or secondary bonds between two antibody molecules attached to the same antigen While no unequivocal evidence of either type of complex exists two findings suggest that complexes other than those considered by Goldberg are important in the antigen antibody reaction (1) the failure in many systems of precipitates to form with an antigen antibody molecular ratio above 0 5 even in the antigen excess zone [3] and ( ) the finding of Singer and Campbell [24] that the total concentration of complexes containing only one molecule of antibody (Ag Ab AgAb and Ab) was less than that predicted by the Goldberg model

The hemolysis of sheep red cells by rabbit antibody has been shown by Mayer and his colleagues [19] to involve a series of reactions with four and perhaps five components [3] of complement An equally complex role for antibody is suggested by findings [33] summarized in Figure 5 The rate of absorption of hemolysin by a standard concentration of red cells is indicated by the first curve on the left This absorption is 50 per cent complete in 5 minutes By assuming that the rate of hemolysis is proportional to the concentration of hemolysin on the cell and by correcting for the lag attributable to the reaction with complement the middle curve in Figure 5 was obtained The lag observed experimentally was much greater than that predicted To explain this lag it was necessary to postulate that the sensitization of red cells by antibody involves more than the simple absorption of antibody by the cell From this finding and a study of the relationship between antibody concentration and hemolytic rate it was possible to construct a model in which the sensitization of cells by antibody required the formation of complexes of two or more molecules of antibody attached to adjoining sites on the red cell According to this model the factors involved in determining the hemolytic efficiency of an antibody were (1) the number and position of the combining sites on the surface of the red cell ( ) the rate of turnover of the antibody on

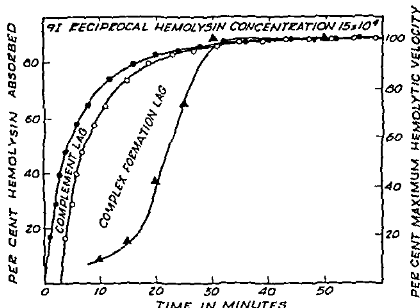
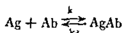


FIGURE 5 Comparison of experimentally observed rate of hemolysin absorption and experimentally observed velocity of hemolysis Solid circles indicate the absorption of antibody the triangles the velocity of hemolysis and the open circles a correction for the lag due to complement absorption

the various antigenic sites (3) the dissociation velocity of the antibody and (4) unknown primary physicochemical properties of the anti body molecule (probably including size) which determine the fixation of complement With this model it was possible to explain the differences in hemolytic efficiency observed with two antibodies both combining with the same antigen on the surface of the cell

With both precipitation and hemolysis an important factor in the outcome is the affinity between antigen and antibody This is a complex *function of the interaction of two molecules* and varies over an extremely wide range I have used the word affinity to express in a very broad sense the relation of antigen and antibody in the reversible reaction



$$Ag + Ab \underset{k_d}{\overset{k}{\rightleftharpoons}} AgAb$$

A free antigen site plus a free antibody site yields a combined site This reaction may be broken down into three components

(1) the velocity of association V_a

$$V = k_a (Ag)(Ab)$$

(2) the velocity of dissociation V_d

$$V_d = k_t (AgAb)$$

(3) the intrinsic dissociation constant K_D which is equal to the ratio of the two rate constants k and k_d

$$K_D = \frac{k_d}{k} = \frac{(Ag)(Ab)}{AgAb}$$

Jerne [8] apparently considered it likely that the rate constant of association was the same for the many different antibodies and that a high degree of correlation existed between the equilibrium constant K_D and the rate constant of dissociation For this reason he used the term avidity to describe an antibody with a relatively small dissociation constant and a slow rate of dissociation Although the correlation between these two functions is undoubtedly high it would be surprising if the correlation were always exact For this reason it seems advisable to avoid the words affinity and avidity except as general terms to introduce an area of consideration

In the model of hemolysis drawn previously changes in any two of the constants (k k_d and K_D) might affect the hemolytic efficiency of the antibody without any change in the third constant Very few attempts have been made to measure the association velocity and its constant k Mayer and Heidelberger [18] presented evidence indicating that the primary reaction between antipneumococcus polysaccharide and a cross reacting antigen was complete in 3 seconds Consequently the rate of reaction was considered too high to measure However the forward reaction velocity unlike the reverse reaction is dependent on concentration and can be measured readily enough if the reactants are sufficiently diluted and if the dissociation constant is such that at the low concentrations required for the study a large fraction of one of the reactants is combined at equilibrium Under these conditions k_a has the dimension of the reciprocal of the concentration of free antigen sites at which half of the antibody sites react in 1 second More specifically, if one of the reactants (e g antigen) is in considerable excess $k_a = \frac{0\ 7}{t_{1/2}C_{ag}}$ where C_{ag} is the concentration of free antigen sites and $t_{1/2}$ is the time required for *half of the antibody to react*

Using the rabbit hemolysin for sheep red cells Weinrach [33] measured the rate of absorption of hemolysin at 37° C in antigen excess and at a concentration of antigen yielding approximately 90 per cent absorption of antibody at equilibrium (Figure 6) The concentration of cells was 6×10^9 per liter which is approximately 10^{15} antigen sites per liter or $1\ 5 \times 10^{-9}$ M The measured $t_{1/2}$ was 300 seconds Thus $k_a =$ $\frac{0\ 7}{300 \times 1\ 5 \times 10^{-9}} = 1\ 5 \times 10^6$ This must be considered as an order

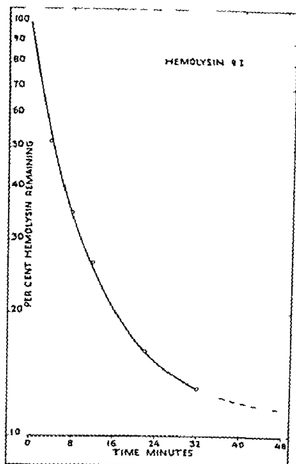


FIGURE 6 Rate of absorption of hemolysin in region of antigen excess

of magnitude approximation since there is uncertainty concerning the number of antigen sites per cell and the valence of antibody which is probably greater than the assumed valence of 1 However a k of the same magnitude (10^5) was obtained with the antibovine serum albumin (anti BSA) and I^{131} BSA using ammonium sulfate to stop the reaction Since the uncertainties concerning valence in these determinations are probably within a factor of 10 it is reasonable to assign 10^5 as the lower limit of k_a in the antibodies studied A k_a of 10^5 means that at a concentration of antibody of 10^{-5} M or 100 μg N/ml (bivalent antibody molecules of molecular weight 160 000) and an equal concentration of antigen sites half of the primary reaction is completed in approximately 1 second At a concentration of 1 μg antibody N/ml half of the reaction is completed in approximately 100 seconds Slower reaction rates between antigen and

antibody have not been described, and this limit is compatible with observations on the kinetics of phage neutralization[3] The limiting value of k_a is emphasized here because it permits the assignment of a limiting value to the relationship between the equilibrium constant K_D and velocity constant k_d for $K_D = \frac{k_d}{k_a}$ Thus, if the half dissociation time of the antigen antibody complex is several days k_d approximates 10^5, and K_D is at least as low as 10^{-10}

The rates of dissociation which are relatively easy to measure with labeled antigens and antibodies provide an additional method of determining the equilibrium constant and a means of checking equilibrium constants obtained by other methods The general method is illustrated in Figure 7 by the dissociation of labeled antibody from sheep red cells Labeled antibody is absorbed by a sheep cell suspension, and after the reaction is complete a very large excess of unlabeled antibody is added which effectively prevents a labeled molecule once dissociated from ever readsorbing The fraction of labeled antibody remaining plotted on a log scale against time permits a determination of the half-time of dissociation In this case $t_{1/2}$ was 3 minutes

$$k_d = \frac{2.3}{t_{½}} = \frac{2.3}{180} = 1.3 \times 10^{-2}$$

$$\text{Upper limit of } K_D = \frac{k_d}{\text{lower limit } k} = \frac{1.3 \times 10^{-}}{10^5} = 1.3 \times 10^{-7}$$

The valence of the antigen or antibody does not enter into the calculation of k_d and no error from uncertainty of valence occurs in this calculation except that already considered in the determination of k_a The dissociation constant of 10^{-7} obtained here with the Forssman antigen of sheep red cells agrees well with the value obtained by the Wurmsers[34] for the reaction between beta isoagglutinins and human type B cells Both involve lipo polysaccharide antigens Using a labeled bovine serum albumin (I*BSA) combined with antibody in far antigen excess where the major part of the bound antigen is in small complexes containing only one antibody molecule the rate of exchange with unlabeled antigen has been measured Half times from several hours to several days were obtained[29] This corresponds to a $t_{1/2}$ of 10^3 to 10^5 seconds and a K_D of 10^{-8} to 10^{-10} or less This agrees well with the determination of K_D by Jerne in the diphtheria toxin antitoxin system

The dissociation constant, K_D is equal to the concentration of free antigen sites at which half of the antibody is bound and also the concentration of free antibody sites at which half the antigen is bound For

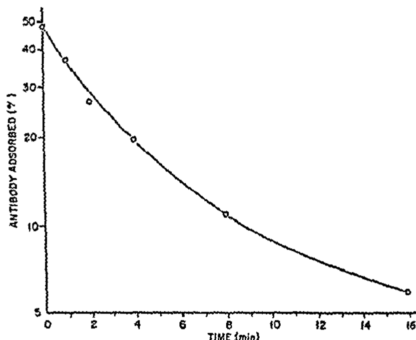


FIGURE 7 The elution of labeled antibody from red cells in the presence of a large excess of unlabeled antibody Half elution time 3 minutes

reasons which are apparent from a study of Figure 3 it is rarely possible to detect an antibody if both antigen and antibody sites are present in a concentration much below K_D This constant therefore provides a rough estimate of the sensitivity of an antigen antibody reaction In Table I this relation between antigen and antibody concentrations and K_D is utilized to indicate the meaning of the values of K_D which have been obtained in various antigen antibody systems

At the upper limit of K_D the values of the equilibrium constants for antigen antibody reactions approach those of nonspecific reactions such as that between methyl orange and serum albumin This upper limit is quite arbitrary and is imposed by the above mentioned fact that antibody cannot be detected if the molar concentrations of both antigen and antibody sites are below K_D For most practical purposes this is near the level of gamma globulin in serum of 10^{-4} M The lower end of the scale is imposed by biological necessity Antibodies with an equilibrium constant much above the toxic level of a given antigen would be relatively inefficient in neutralization Only by a large excess of free antibody can the concentration of free antigen in such a system be forced down below

TABLE I THE RELATIONSHIP BETWEEN THE EQUILIBRIUM CONSTANT AND THE RATE OF DISSOCIATION

K_D M/liter	Antigen System	Concentration Equal to	$t_{1/2}$ of Dissociation Assuming $k_a = 10^5$
10^{4}	Dye albumin [10]	globulin in serum	0 07 sec
10^{5}	Haptens [4 6 16]	100 μg Ab N/ml	0 7 sec
10^{6}			7 sec
10^{7}	Isoagglutinins [34] Forssman antigen	1 μg Ab N/ml	1 2 min
10^{8}			12 min
10^{9}	Bovine albumin Diphtheria toxin [8]	01 μg Ab N/ml	1 9 hr
10^{10}			19 hr
10^{11}			8 days
10^{1}	Toxic level of diphtheria toxin	1 molecule/cell	80 days

The first column indicates the dissociation constant K_D The second column gives the various antigen antibody systems which have been studied adjacent to the equilibrium constants that have been obtained In the third column these are compared with the concentrations familiar to the immunologist The fourth column gives the upper limit of the half time of dissociation based on the assumption that k is 10^5

the toxic level In the case of toxin neutralization therefore the equilibrium constant is a major factor in determining the efficiency of the antibody in the neutralization process

The wide range of equilibrium constants which have been found for various antibodies as listed in this table illustrates the importance of this constant in antigen antibody reactions Although there is a ten million fold difference between the fastest and slowest dissociation velocity this may represent only a little over a twofold difference in strength of bond between the two molecules The transition from highest specificity to nonspecificity is gradual with no sharp line of separation Since nonspecific affinity between substances is so common the structural complementariness of antigens and antibodies is after all not so remarkable This raises the question we started out to answer Just what is an antibody?

ACKNOWLEDGMENTS

I wish to acknowledge helpful discussions with Professor D H Campbell and Professor V Schomaker of the Division of Chemistry and Chemical Engineering of the California Institute of Technology Pasadena California during a three month leave spent in Professor Campbell's laboratory

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2

*Quantitative Measurement of Heterogeneity among Antibodies**

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(Philadelphia Pennsylvania)

INTRODUCTION

The heterogeneity of antibodies has been a recurrent theme in immunology for many decades There are a number of variations of this theme which have emerged from problems concerned with the stimulation and mode of production of antibody Among the most important kinds of heterogeneity, either for practical or theoretical reasons are those arising from the multiplicity of antigens from the presence of several antigenic groups on a single protein molecule and from the variation in the degree of complementarity of antibody sites directed against a single antigenic group Our discussion will be concerned with the last kind of heterogeneity which represents from the molecular structural point of view the most subtle aspect of the variability of the immune response The study of variable complementarity also bears on the problem of the mechanism of antibody formation since it is apparent that an acceptable theory of this process must account not only for specificity – that is complementarity – but also for its variability

Our approach to the problem of variable complementarity has been through the use of purified antihapten antibodies These macromolecules form soluble complexes with monohaptenic dyes carrying the homologous haptenic group The quantitative correlation between the fraction of antibody sites occupied and the concentration of free dye over a wide range of the latter provides the experimental basis from which inferences can be drawn about the heterogeneity of the antibody sites

EXPERIMENTAL SYSTEMS AND RESULTS

Two antibody hapten systems have been used in our work The haptenic groups involved and the related azohaptenic dyes are shown in

* These studies were aided by a grant from the National Science Foundation and by a research grant (H 869) from the National Heart Institute of the National Institutes of Health U.S Public Health Service

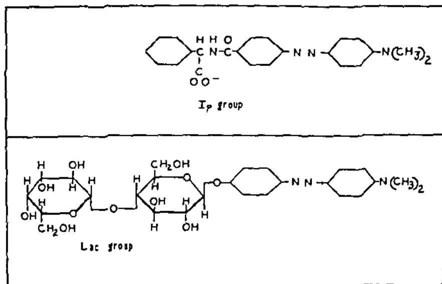


FIGURE 1 Formulas of the haptenic groups and the corresponding dyes

Figure 1 These groups are p-azophenyl-β-lactoside, designated as the Lac group, and phenyl (p-azobenzoylamino) acetate, designated as the I_p group. The latter group consists of two optically isomeric forms which are referred to as D-I_p and L-I_p. The dyes themselves are known as Lac dye and D-I_p or L-I_p dye. The original observations of the antigenicity of the Lac group were made by Goebel, Avery, and Babers[1] and of the I_p groups by Landsteiner and van der Scheer. The Lac group differs from the I_p group in the two important respects that it is uncharged whereas the latter is anionic and that its main portion has a strong affinity for water unlike the neutral form of the I_p group.

The purification of the rabbit antihapten antibodies was carried out by a previously described procedure[8] involving the specific precipitation of the antibody with an antigen consisting of hapten coupled to human fibrinogen. The immunizing antigen consisted of hapten linked to bovine gamma globulin. By virtue of the initial precipitation step and the incomplete recovery (50 per cent) of the precipitated antibody in the final preparation of purified antibody, there is introduced an artificial selection of the antibody whose heterogeneity is being evaluated. The effect of this selection is to exclude antibody with the largest variations from the average value of the association constant for the antibody-hapten complex. Antibody of small affinity may be excluded at the initial precipitation, although this tendency has been minimized by the use of a precipitating antigen of low solubility. Antibody of relatively high affinity may be eliminated at the extraction step of the purification in which most of the antibody contained in a specific precipitate is brought into solution with

our solubilizing the antigen Although the quantitative significance of this selection is not known it is very likely that the final preparation of purified antibody will be less heterogeneous than the antihapten antibody initially present in the antiserum

There is an additional source of uncertainty in our antibody hapten systems which arises from our inability to define precisely the effective antigenic group associated with the haptenic group which is linked to the protein of the immunizing antigen This limitation is due to the possibility that the azo linked residues histidine and tyrosine and neighboring residues are part of the antigenic group However, the energetic significance of such residues in the formation of antihapten antibody which is precipitable with the hapten fibrinogen is probably small We base this surmise on the observation that the terminal portion of the haptenic group exercises the dominant role in the interaction between the antibody and hapten[4]

The combination of antibody and the azohaptenic dyes (Figure 1) which carry the homologous haptenic groups was investigated by the method of equilibrium dialysis[3, 4] The systems studied lend themselves readily to quantitative interpretation because nonspecific free energy contributions arising from phase transition and from protein protein interactions are absent The intense absorption bands of the dyes allow accurate measurements of concentrations in solutions as dilute as 10 M The antibody concentrations employed ranged from $\times 10^{-}$ M to 3×10 M and with total dye concentrations of the same order of magnitude most of the dye was in complex form Under these conditions an accurate correlation between fraction of antibody sites occupied and free dye concentration can be obtained

Binding curves for both systems obtained at two temperatures are shown in Figures and 3 The experimental results are plotted in the form of r/c *vs* r where r is the average number of dye molecules bound per antibody molecule at the concentration c of the free dye This kind of plot is particularly useful for our purpose since the deviation from linearity provides a measure of the heterogeneity of the combining sites with respect to their affinity for the azohaptenic dyes This can be seen from an expression which is readily derived from the law of mass action for the case in which all the antibody sites have the same intrinsic association constant K In this event

$$r/c = nK - rK \qquad (1)$$

where n is the valence or number of combining sites of the antibody molecule The value of n is easily obtained from the r/c *vs* r plot by extrapolation of the experimental curves to the abscissa For both kinds of antibody a value of 0 for n is thus found

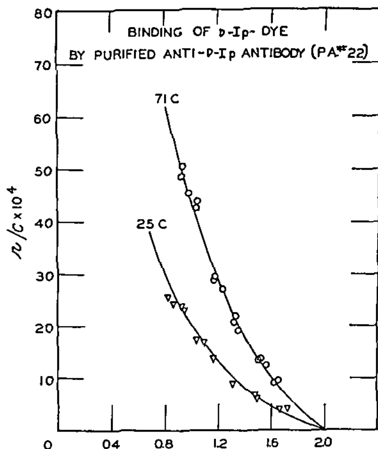


FIGURE 2 Binding results at 25 and 71 for the reaction between D I dye and purified anti D-I antibody The points are experimental and the curves are theoretical (Reprinted from *Journal of the American Chemical Society* with permission see reference 3)

ANALYSIS OF HETEROGENEITY

Examination of Figures 2 and 3 shows that the antibody sites in both systems are heterogeneous since the binding results deviate substantially from linearity This heterogeneity can be given quantitative expression by using the assumption first formulated by Pauling *et al*,[8] that the distribution of the antibody affinities can be described by the normal Gauss distribution in terms of the standard free energy of binding ($\Delta F°$) as follows

$$a(\Delta F°) = \frac{1}{\sigma\sqrt{\pi}} e^{-(\Delta F_0° - \Delta F°)^2/(RT\sigma)^2} \qquad (2)$$

in which $\Delta F_0°$ is an average standard free energy corresponding to the maximum in the distribution function The degree of heterogeneity is

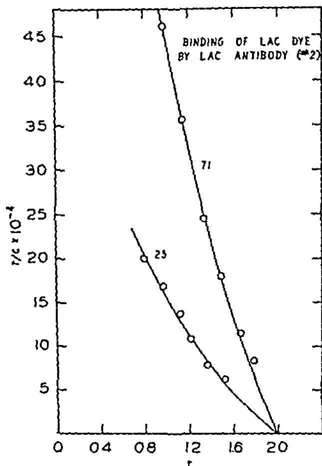


Figure 3 Binding results at 25° and 7.1° for the reaction between Lac dye and purified anti Lac antibody. The points are experimental and the curves are theoretical (Reprinted from *Journal of the American Chemical Society* with permission see reference 4.)

given by σ the heterogeneity index and the other symbols have their usual meaning. The distribution can be expressed in terms of affinity constants by using the expression $-\Delta F^\circ = RT \ln K$ to yield

$$u(K) = \frac{1}{\sigma\sqrt{\pi}} e^{-[\ln(K/K_0)]^2/\sigma^2} \tag{3}$$

The fraction of the total number of sites which have K values in the infinitesimal region K to $K + dK$ is given by

$$\frac{dN}{N} = \frac{1}{\sigma\sqrt{\pi}} e^{-[\ln(K/K_0)]^2/\sigma^2} \, d[\ln(K/K_0)] \tag{4}$$

With the aid of this relation and the assumption that the sites act independently it can be shown that the fraction of sites occupied as a function of c is expressed by

$$r/n = 1 - \frac{1}{\sqrt{\pi}} \int_{-\infty}^{\infty} \frac{e^{-\alpha^2}}{1 + K_0 c e^{\alpha}} \, d\alpha \tag{5}$$

in which α has been substituted for $\ln(K/K_0)/\sigma$ Since this integral cannot be evaluated analytically we have carried out numerical integration with the aid of the Gauss quadrature formula as described by Greenwood and Miller[2] It turns out furthermore that when $K_0 c = 1$ the value of $r/n = \frac{1}{2}$ for all values of σ This means that the reciprocal of the free concentration of dye c at which one half of the antibody sites are occupied is the value of K_0 In this way the values of K_0 shown in Table I have

TABLE I Binding of Azohaptenic Dyes by Homologous Purified Antihapten Antibody

			25		7 1			
Dye	n		$K_0 \times$ 10^{-}	$-\Delta F$ kcal / mole	$K_0 \times$ 10^{-5}	$-\Delta F$ kcal / mole	$-\Delta H$ kcal / mole	ΔS e u / mole
D I_t	2 0	2 3	2 05	7 25	4 4	7 24	7 1	0 3
Lac	2 0	1 5	1 57	7 09	4 48	7 25	9 7	−8 8

been obtained from the experimental binding curves The corresponding values of the free energy of binding for both temperatures are also shown as well as the quantities ΔH and ΔS derived from the temperature dependence of K_0 The significance of these changes in enthalpy and entropy has been discussed elsewhere[3, 4]

In order to establish the adequacy of equation (5) for the description of the experimental results theoretical binding curves were calculated for judiciously selected values of σ using the experimentally established values of K_0 and n and the laborious procedure of numerical integration indicated above The values of σ shown in Table I yielded theoretical curves that agreed within experimental error with the experimental results This can be seen in Figures and 3 in which the points are experimental and the solid lines are the theoretical binding curves It should be understood however that since low values of r were not experimentally accessible it cannot be concluded that a Gaussian distribution provides a complete description of the heterogeneity of our purified antibody preparations Nevertheless in the absence of further information we may tentatively employ the Gaussian distribution as a quantitative basis for the analysis of variable complementarity

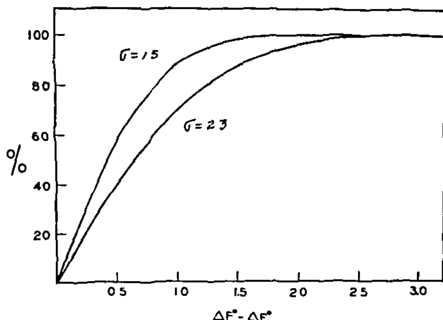


FIGURE 5 Plots of the integrated form of the distribution function $w(\Delta F)$ for two values of σ

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3

*Heterogeneity of Diphtheria Antitoxin**

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It is a matter of common knowledge that filtrates from cultures of diphtheria bacilli used for the preparation of crude diphtheria toxoids contain, besides the specific toxin, numerous antigens (which will be designated in this paper as "accessory diphtheria antigens"). It is clear that sera of animals immunized with crude toxoid must contain, together with the antitoxin, antibodies acting against these accessory antigens. In fact, they have been observed in man (Kuhns and Dukstein[38]), but it is in horses that they are most clearly seen, because of the long hyperimmunization these animals are being exposed to for the preparation of therapeutic sera.

The heterogeneity of sera from animals immunized with crude toxoids is widely recognized. It does not raise special theoretical problems and would not deserve special emphasis if it were not for the discussions that have been stirred up on the characteristics of the pure toxin[55, 6, 64]. There were great difficulties in the assessment of the number of accessory antigens and antibodies before the use of Oudin's methods of specific precipitation in gels[5, 54] and the numerous developments of these techniques[49-51]. It is now easy to verify the extreme complexity of crude or partially purified diphtheria antigens and of commercial sera[4, 61, 64, 81]. Precipitation curves established with complex crude or partially purified toxins cannot be considered as being related to the toxin-antitoxin system only, but may be the result of a superposition of many systems. Using a pure toxoid of 3000 Lf/mg N containing only a single detectable antigen, one can obtain in the horse sera which have a single antibody, the precipitating antitoxin[6, 70]. However, if the immunization is extended for a very long time, traces of accessory antigens contained in these preparations

* The antidiphtheric antitoxin is defined as "an antibody which can combine specifically with the diphtheria toxin taken as a homogeneous protein. In the toxin-antitoxin complex the toxic activity of the toxin may be or may not be neutralized. The specificity of the antitoxin is not obligatorily associated, therefore, with its ability to neutralize the diphtheria toxin, but with its ability to combine specifically with the toxin. It is the same as the definition which is accepted for antibodies directed against other proteins devoid of a specific biological activity."

of high purity give rise in the long range to supplementary antibodies[9] This does not impair the identification of the toxin antitoxin system which remains quite easy in a complex system thanks to the use of pure toxin as a detecting agent by specific precipitation in gel (Figure 1) or by

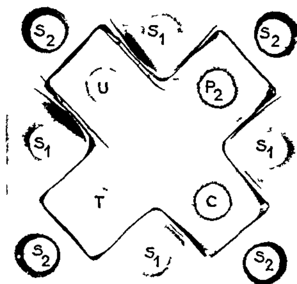


FIGURE 1 Preparations of increasing purity obtained in the process of purification of diphtheria toxin Fraction U 00 Lf/mg N Fraction P 1150 Lf/mg N Fraction C 2000 Lf/mg N Purified toxin 3000 Lf/mg N Each preparation is tested against a complex serum (S_1 = 906 serum) and against a one zone serum (S = 1451 bis)

immunoelectrophoresis[80] Even with these pure systems in which precipitation of the antitoxin gives a single line in gelified medium important differences are found in antitoxin properties This heterogeneity of antitoxin manifests itself by differences which we have classified under four headings (1) differences in the electrophoretic mobility ( ) differences in precipitation properties (3) qualitative differences in neutralizing power (avidity) (4) differences in the mode of reaction of antitoxins with a partially digested toxin

DIFFERENCES IN THE ELECTROPHORETIC MOBILITY

In most animal species the antidiphtheria antibody is like other antitoxins a gamma globulin (gamma 1 globulin) * However Kuhns[3] has

* Some authors (Deutsch and Nichol[16]) designate ordinary slow moving gamma globulin as gamma 2 globulin The globulins of intermediary mobility specially abundant in horses hyperimmunized against toxins[63 56] are called by these authors gamma 1 globulins We call these last ones beta 2-globulins in agreement with Kekwick[3 31] and Cinader and Weitz[13 14] After Boyd[8] one may consider that beta 2 = gamma 1 = T

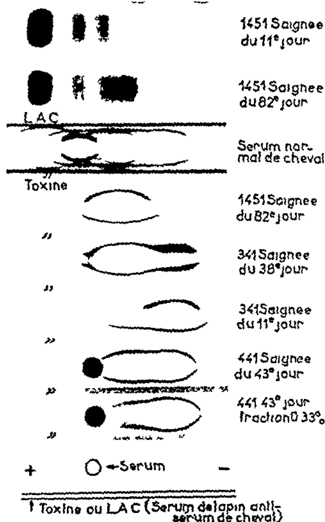


FIGURE Immunoelectrophoretic patterns of serums obtained at different stages of immunization of horses with purified diphtheria toxoid Electrophoresis 4 hours I = 45 mA and V = 70 volts Detection by pure (one zone) diphtheria toxin The concentration of toxin for detection has been chosen according to the titer of serum

shown that nonprecipitating sensitizing antitoxins in man are beta globulins (gamma 1) In hyperimmunized horses the precipitating antitoxin is at the end of immunization a beta globulin On the contrary, in the beginning of hyperimmunization even in the horse the antitoxin is a gamma globulin (Kekwick [82, 83] Relyveld *et al* [9])

These facts can be established by free electrophoresis (before and

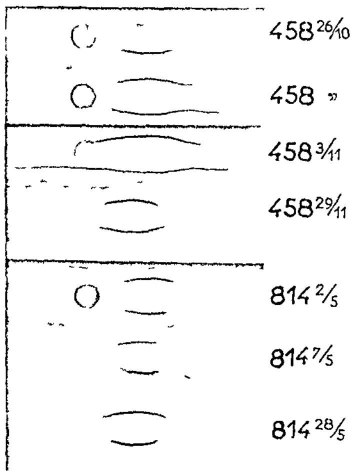


FIGURE 3 Immunoelectrophoresis of sera obtained at different stages of immunization of horses with a purified tetanal toxoid Electrophoresis 4 hours I = 45 mA and V = 70 volts Detection by pure (one zone) tetanal toxin The concentration of toxin for detection has been chosen according to the titer of serum

after absorption of the diphtheria antitoxin with the specific toxin) by electrophoresis on inert support or by immunoelectrophoresis [1, 80]

We resorted to that last technique in order to follow mobility variations of antitoxic antibodies during hyperimmunization of horses against diphtheria toxin [73, 9] (see Figure 2) and against tetanal toxin [74] (see Figure 3)

In some horses immunized with a purified tetanal toxoid (Horse 458) we observed a localization of the antitoxin in alpha globulins This effect was only perceptible during the intermediary period of hyperimmunization and did not last more than a short time slightly before the time when beta 2 antitoxins become entirely predominant

Differences in Precipitation Properties

Antitoxic antibodies like many other antibodies can be precipitating or nonprecipitating

Precipitating Antitoxic Antibodies

It is known from the work of Heidelberger and co workers [5] and of Pappenheimer and co workers [13] [87, 8] that, with the serum of most animal species the toxin antitoxin precipitation curve (or more generally the protein antiprotein precipitation curve) is bell shaped (precipitin type) With the horse's serum, however the curve has a peculiar shape (flocculation type) characterized by the solubility of the specific precipitate in antibody excess and the existence of a long equivalence zone

We were able to establish by the use of sera prepared by immunization of horses with pure diphtheria toxoid that both curves can be found in these animals and are related to the electrophoretic nature of the antibody [73, 77] Gamma antitoxins even in horses give precipitin type curves beta antitoxins give flocculation type curves (Figure 4)

The equivalence zone is actually long with beta antitoxins especially when the excess of antigen or antibody is searched for in supernatants by

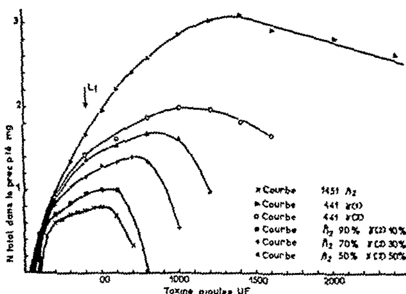


Figure 4 Precipitation curves of different mixtures of beta 2 antitoxins (prepared from 1451 serum) and gamma antitoxin (prepared from 441 serum) Antitoxin concentration 400 Lf unit Total volume 5 ml Incubation 37 1 hours and 0 2 days Buffer NaCl 0 140 M containing borate 0 01 M pH = 7 2 Total nitrogen of washed precipitates has been determined by the micro-Kjeldahl method

precipitation reaction It is on the contrary reduced to a very short zone around L+ point if one is looking for antigen and antibody in excess by titration *in vivo* (Bowen) and even more when the nonneutralized toxin is searched for not only in supernatants but also in specific precipitates It is found then that as soon as the ratio antigen antibody is higher than the L+ value the specific precipitate contains a certain amount of non neutralized toxin (Raynaud and Relyveld [76 77]) The L+ point situation on the curve is very variable when compared with the maximum precipitation point It is localized in the zone of great antibody excess for low-avidity sera When antitoxin is digested by pepsin its electrophoretic mobility is diminished but the general shape of the precipitation curve is not modified Digested beta antitoxins (which we shall call beta prime antitoxins) exhibit a mobility which is almost the same as that of ordinary gamma globulins but they give a flocculation type curve The solubility of the specific precipitate in excess of antibody can be suppressed when the specific precipitation is carried out in concentrated salt medium [60] but the long lasting lineary zone is preserved [2] (see Figure 5) In man as well as in rabbits the curve is usually of the pure precipitin type [15] We know that it is a gamma antitoxin

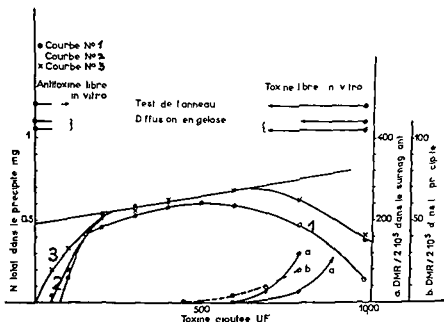


FIGURE 5 Quantitative precipitation curves of a digested beta 2 diphtheria antitoxin prepared from 1451 serum ○ Curve 1 buffer NaCl 0 140 M containing borate 0 0 M pH = 7 ● Curve 2 buffer 1 0 M PO_4KH /K H pH = 7.0 × Curve 3 buffer 1.4 M PO_4KH /K H pH = 7 0 Other conditions as in Figure 4

Nonprecipitating Antibodies

It is known that antitoxic neutralizing nonprecipitating antibodies are encountered They have been studied mostly in man Kuhns[35] has shown that in man they belong to two different types The first are nonprecipitating neutralizing nonsensitizing and are associated with gamma globulins (gamma 2 globulins) the others are nonprecipitating neutralizing and skin sensitizing and are related to beta globulins (gamma 1 globulins) They are met with most often in individuals with an allergic background Extensive studies have been carried out on them by Kuhns and Pappenheimer and their co workers[34 41]

Neutralizing nonprecipitating antibodies have been detected sometimes in horses Barr and Glenny[4] described a serum of that type Neutralizing nonprecipitating antitoxins are rarely found alone in horses In a general way they are associated with precipitating antitoxins When they exist in high concentrations the serum of the horse has abnormally high values of the ratio L+/Lf (see for example Horse 379[4] —L+/Lf = 3 14 to 5 06) A low salt fraction of that serum had even a ratio L+/Lf = 18 9*

We studied in horses nonprecipitating antibodies by using a pure toxin labeled with I^{131} following a method analogous to Masouredis's[47 49] by which both toxicity and combination power of the toxin are not impaired The use of pure labeled toxin allows one to determine directly the presence of nonprecipitating antibodies independently from determination of the neutralizing power We found that in hyperimmunized horses nonprecipitating antitoxic antidiphtheria antibodies are more abundant in the beginning of hyperimmunization It is a well known fact observed with other antigens The maximal levels we found in our horses are 30 per 100 (arbitrarily expressed as the ratio labeled toxin in supernatant to total added toxin)

When sera with gamma + beta 2 antitoxins are fractionated it is found that nonprecipitating antibodies are relatively more abundant in the low salt fraction (SO Am 0 to 0 33 saturation)

* In connection with this observation the following remark should be made It is well known that by aging antitoxic sera may lose their flocculating power while keeping their neutralizing power Such a type of nonprecipitating character has not to be obligatorily assimilated to the nonprecipitating character observed in fresh sera The disappearance of the precipitating power by aging corresponds to some change in the globulin molecule Similar changes may happen as we observed by repeated fractionation procedures using concentrated neutral salts and dialysis Gamma antitoxins seem to be from that point of view more instable than beta antitoxins One must therefore be careful in interpreting the fractionation experiments The high levels of nonprecipitating antibodies observed in low salt fractions do not correspond obligatorily to the real levels of the nonprecipitating antibodies in the native serum They may originate from artificial modifications supported by gamma globulins during fractionation procedures

By peptic digestion of beta antitoxins especially when the digestion is effected on the specific precipitate and not on whole serum (beta antitoxin toxin) digested antitoxins (beta prime antitoxins) are obtained which are almost entirely devoid of nonprecipitating antibodies. This has been also observed by Pappenheimer and Yoneda [9] in peptic digested whole sera.

Qualitative Differences in Neutralizing Power

Antitoxins produced during the first stages of immunization in individuals devoid of basic immunity have an irregular neutralizing power. It is said that these antitoxins have a low avidity. Complexes between toxins and these low avidity antibodies are easily dissociated by dilution so that the precise determination of the neutralizing power is related to the absolute concentration of the reagents (titration level). The abnormal characteristics of antibodies with low avidity have been described in great detail by Glenny and Barr [13, 17, 18] and by Jerne [28, 31].

One can obtain high concentrations of antibodies of that kind (with low avidity) in sera by immunizing with a pure toxoid adsorbed on calcium phosphate Schick positive horses devoid of basic natural immunity.* As was already pointed out by English workers such sera have a very low L+/Lf ratio (0.30 to 0.50). We prepared a certain number of such sera and established their quantitative precipitation curve.

The most notable property of these sera is the following: the specific precipitate which is devoid of toxicity towards large excess of antibodies becomes toxic starting from L+ point situated in that case much before the point corresponding to the maximum of the precipitation curve. This phenomenon is very similar to what is observed with certain enzyme antienzyme systems [91].

The dissociation between neutralizing and precipitating powers observed under these conditions is only transitory and disappears when immunization is lengthened. In our opinion it has a great theoretical interest.

One can consider of course that toxicity of specific precipitates is the sole result of the weakness of linkage between low avidity antitoxins and toxin which renders possible the dissociation of the specific precipitate in the animal's body. It is the theory usually accepted.

* Since the observation of Hitchens and Tingley [26] and Sordelli [8] confirmed by other authors [19, 20, 67, 69] it has become usual to employ only horses presenting basic immunity for the production of commercial sera. This basic immunity may be "artificial (tetanus) or natural (diphtheria). The artificial basic immunity is obtained by a preliminary vaccination followed by a long period of rest. Barr and Glenny [5] have intensively discussed the reasons for this selection. Consequently almost all the commercial sera are avid and do not present the dissociation between precipitating and neutralizing powers—*a dissociation which we discuss later on.*

However another hypothesis can be formulated The different antigenic groups which are supposed to be present on the molecule of diphtheria toxin are not all equivalent Some of them can combine with the antibody without neutralization of the toxin We shall call such antigenic groups t_1 t_2 t keeping the symbol t for groups able to give rise to neutralizing antibodies in the animal receiving the whole toxin (T)

The 't group is not necessarily toxic by itself We shall see on the contrary that after a partial proteolytic digestion this group is associated with a part of the molecule devoid of toxicity but able to combine with neutralizing antibodies This fact is not very surprising if it is remembered that there are similar antigenic groups on toxoid groups without toxicity but able to produce in the animal neutralizing antibodies

t_1 t_2 t groups give rise to a_1 a a antibodies which can combine specifically with the toxin without neutralization

Let us examine the arguments in favor of that hypothesis

Differences in the Mode of Reaction of Antitoxins with a Partially Digested Toxin

a a_1 a a distinct antibodies produced by injection to animals of a unique T antigen (diphtheria toxin) carrying t t_1 t t antigenic groups can be observed by studying reactions between antidiphtheric sera and digested toxin using Lapresle s technique employed by that worker for human serum albumin and human serum antialbumin system[4, 46] Pope[1] by a similar technique has shown that if a diffusion against an antidiphtheric serum is made with a pure toxin on the one hand and partially digested toxin on the other hand starting from two separated centers (Ouchterlony s technique) many precipitation lines are observed with the digested toxin each of them giving an identity reaction with intact toxin With sera used by Pope three different lines were obtained

In Pope s opinion it is the proof of the heterogeneity of the antigen (toxin) whereas we think it is only due to the heterogeneity of immunity reaction (presence of different antibodies a a_1 a a) evoked in the animal by injection of a single antigen

We have studied our sera by the following method[73] First we determined the most favorable conditions of proteolytic digestion for the characterization of such multiple systems (Table I) The toxin digested for 1 hour at 37 C at pH = 7 (0 11 µg crystallized trypsin per toxin Lf unit) was used for diffusion gel experiments With avid sera (441 906 1451) we observed two very strong lines and a very weak one (this last is not visible in the photograph Figure 6) With a nonavid serum (825)

TABLE I

Serum Number	L+ Lf	UF mg N	Per Cent of Precipitable N	Immunoelectrophoresis		Reaction with Digested Toxin*		
				γ	β	a	a_1	a
906	0 86	108	15	β′		+++	++	[illegible]
825 (39th day)	0 32	47 5	8 45	++	++	+	+++	
441	1 87	17	7 68	+++	++	+++	++	[illegible]
1451	0 85	45	6 80	0	+++	+++	+++	[illegible]
341	0 90	84	17 60	+	+++	+++	++	[illegible]

The several antibodies a a_1 a_2 have been identified by absorption experiments

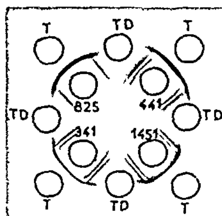


FIGURE 6 Reaction in gel between partially digested diphtheria toxin and several horses sera Pure toxin is indicated by T and digested toxin by TD The numbers are those of the sera

we observed one strong line and a very weak line (this last not visible in the photograph)

By absorption experiments we were able to establish that the strong line in nonavid serum (825) corresponds to nonneutralizing antitoxin

The following experiments are summarized in Figure 7 First we absorbed a digested toxin with a nonavid serum (825) We eliminated by this procedure the majority of antigens The absorbed digested toxin (TDE) then contained only one of the several antigens normally present in digested toxin TDE reacting with an avid serum (906) eliminates by precipitation its neutralizing antibodies The resulting exhausted serum (906 E) gives now one line only with digested toxin (TD) This line is identical with the one present in nonavid sera (we call it a_1) The line which disappeared is probably the line corresponding to the neutralizing antibody (which we call a)

We established also precipitation curves of these sera with digested toxins (Figures 8A 8B 8C and 8D)

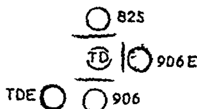


FIGURE 7 Absorption experiments controlled by specific precipitation in gel 825 normal horse serum 906 acid complex serum TDE digested toxin absorbed by 825 serum 906 E 906 serum absorbed by TDE TD digested toxin

In the chosen type of digestion the flocculating power of the digested toxin is reduced by one half (the measurement is made with the flocculating standard) Units used in abscissa are real flocculation units and therefore their amount in curves of digested toxins is equal to flocculation units of the original toxin

One can also see that in that case different sera do not give the same results Serum 8 5 always presents a peculiar behavior the differences between curves plotted for intact and digested toxins are much more pronounced than for other sera

We made the same experiments on the tetanus toxin antitoxin system (Table II) We have also determined the most favorable conditions for proteolytic digestion (toxin concentration 3000 Lf/ml trypsin 40 μg/ml pH = 7 37 C for 1 hour)

We proceeded then to the study of different sera by specific precipitation in gel

Patterns of these sera and the results obtained are given in Table III

In our opinion these data are difficult to explain with the *sole* help of the usual concept of avidity as it has been defined by Jerne [9] because it implies more or less implicitly the existence of a family of antibodies and molecules with affinity constants of *different values* for the *same* antigenic group

The notion which I shall now discuss does not exclude by any means the existence of possible differences in affinity between an antibody and its antigen considered as being unique It introduces the action on the global avidity of a serum of another factor the definition of which shall be given namely the balance of an immun serum towards a given antigen

We call balanced sera the ones in which antibodies corresponding to the different antigenic groups of the toxin molecule (or more generally of the antigen) are present in a proportion which corresponds approximately to the *relative* frequency of these groups on the whole protein molecule

In balanced or avid sera the different systems $a - t$ $a_1 - t_1$ $a - t$

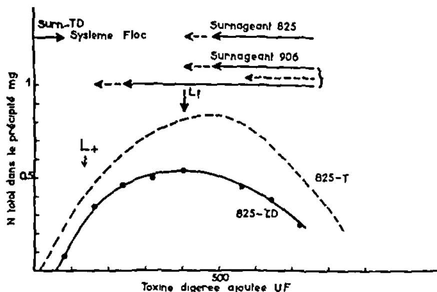


FIGURE 8A

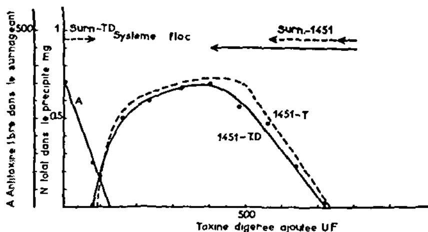


FIGURE 8B

are at equivalence at the same time This means that the different systems are at the equivalence for the same ratio antigen/serum

Nonavid sera are on the contrary nonbalanced or disequilibrated sera This means that the systems a — t $a_1 - t_1$ a — t are not at equivalence for the same values of the ratio antigen/serum

A nontoxic complex or combination of toxin with a nonavid serum in this case must contain an excess of nonneutralizing antibodies The

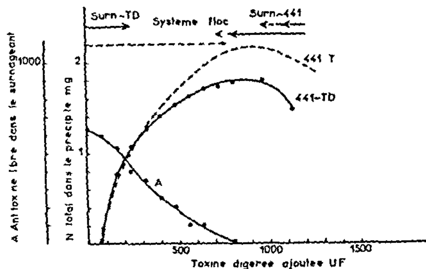


FIGURE 8C

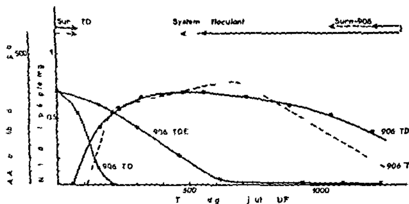


FIGURE 8D

Precipitation curves of different antidiphtheric horses sera with intact toxin (T) and digested toxin (TD)

special composition of this complex can explain the characters of a nonavid serum

The neutral mixture corresponding to L point is situated in the region of a large excess of antibody (see Figure 8A) which must be therefore the nonneutralizing antibody The formation of this complex depends then on the concentration of this nonneutralizing antibody (which we have called a_1)

TABLE II Digestion of Purified Tetanal Toxin

Time	Lf ml	MLD/ml 10³	Number of Lines
0 hour	3000	66	1
1 hour	?750	60	2
3 hours	?000	30	2
6 hours	2000	20	2
7 hours	2000	20	2
24 hours	1500	20	2

Concentration of toxin 3000 Lf/ml concentration of crystallized trypsin 0 48 μg/ml pH = 7 2 temperature 37 C

At the indicated time one determines flocculation titer and residual toxicity (mice minimal lethal dose)

TABLE III Characters of Different Antitetanic Horses Sera

Serum	Prelimi nary Vacci nation	Day of Bleeding	Lf ml	L+ Lf	Immunoelectro phoresis β_2	γ	Reaction with Digested Toxin No 1	No 2	No 3
814	0	23d	656	0 91	+++	±	+++	+	
814	0	44th	1250	1 40	+++	0	+++	+	
815	0	23d	277	0 27	++	++	+	+++	
815	0	44th	5?6	0 27	++	++	++	++	
545	+	23d	184	2 50	+++	0	++	++	
545	+	37th	1310	1 07	+++	0	++	++	
546	+	19th	372	1 80	+++	±	+++	0	
546	+	37th	2650	1 30	+++	±	+++	+	
458	+	24th	2?5	0 70	+++	++	++	+	±
458	+	58th	12?0	1 20	+++	0	++	+	±
458	+	61st	1200	1 40	+++	0	++	+	±
AT	+	—	660	2 40	+++	0	+++	0	
Am	+	—	1800	2 10	β		++	++	
SE	Diluted solution		250	1 00	+++	0	+++	0	

Most of the sera 814 815 545 546 458 have been obtained by immunization of animals with pu anal toxoid The others AT and Am are commercial sera — AT a native Am a peptic digested seru is the International Antitetanal Flocculation Standard

This hypothesis following which low avidity sera are nonbalanced sera can explain in a very simple way the essential characteristics of low avidity sera namely, (1) the dependence of the end point titration (L+ or L) on the absolute concentration of serum (2) the reactivation by dilution of neutral mixtures

There remain of course many obscure points in this theory The isolation under a homogeneous immunological form of fragments of

the toxin molecule carrying each group t t₁ t will allow a precise titration of the level of a a₁ a antibodies

The demonstration of that hypothesis will also be achieved when it is possible to realize for instance the conversion of a low avidity serum into an avid serum by partial absorption with the appropriate antigen

Finally the mathematical formulation of these reactions remains to be calculated

Conclusions

Antidiphtheric antitoxic antibodies obtained from horses can have quite different properties together with the power to combine specifically with the toxin

(1) They can be precipitating or nonprecipitating Precipitating antibodies can give a precipitin type or a flocculation type curve Nonprecipitating antibodies can be sensitizing or nonsensitizing

( ) They can be gamma- or beta globulins

(3) *They can be avid or nonavid*

(4) They can be neutralizing or nonneutralizing

These different properties are sometimes correlated Thus for precipitating antibodies gamma antitoxins give a precipitin type curve and beta antitoxins a flocculation type curve

(5) The reaction type of antidiphtheric sera with a trypsin partially digested toxin shows that there is another kind of heterogeneity Like most proteins the diphtheria toxin contains numerous separate antigenic groups (t t₁ t) One can formulate the hypothesis that one of them only (t) gives rise to neutralizing antibodies which can depending on the conditions be nonprecipitating or precipitating Antibodies specifically directed against each of the antigenic groups are not always in the same relative proportion in all sera

In avid sera the two main systems precipitating neutralizing antibodies and precipitating nonneutralizing antibodies appear to be in approximately equal proportions that is both systems are at equivalence for the same value of the ratio whole antigen/serum We suggest that such sera be called balanced sera

In precipitating nonavid sera both systems are not at equivalence at the *same time the level of neutralizing antibodies being much lower than* the level of nonneutralizing antibodies We suggest calling such sera nonbalanced sera

Acknowledgments

The experimental work of which some unpublished results are given in this paper has been done in collaboration with Mr E H Relyveld for the diphtheria toxin and with Mr A Turpin for the tetanal toxin Studies

on the diphtheria toxin antitoxin system constitute the partial fulfillment of a Ph D dissertation by Mr F H Relyveld presented at the University of Paris

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DESIGNATED DISCUSSION

WILLIAM C BOYD (Boston, Massachusetts) In view of the crowded program it occurred to me that the thing this audience would appreciate most from a discussant is brevity so I have only two remarks to make

One is that I was a little surprised that there was practically no mention of the role of the individuality of the animal in determining what kinds of antibodies can be produced I think this is probably a very important thing and I hope it will not be overlooked In my experience different animals — different rabbits for instance — vary a great deal in the kind of anti bodies they yield and in how much heterogeneity will be seen in the antibodies in a given serum

Another point concerns Dr Karush s paper Many recent workers have reported values for the entropy of reaction ΔH as being practically zero or very small This stands in disagreement with an early result that I published reporting a fairly large ΔH determined experimentally I am glad to see that the ΔH s of Dr Karush are substantial (although still not as large as mine)

WILLIAM J KUHNS (Pittsburgh Pennsylvania) I will discuss our experi ence with the human responses to diphtheria toxoid * An understanding of responses to diphtherial antigens is important in applying this system to studies of immediate type allergies

In primary immunization to toxoid and in secondary immunization where multiple closely spaced doses of toxoid are given precipitins are ordinarily not measurable and the only feasible form of calibration is the rabbit neutralization test [4] or a similarly sensitive hemagglutination test using modified red blood cells [1] A single booster dose of toxoid given subsequent to primary immunization often stimulates the formation of precipitins in persons who form high neutralizing titers [2, 3] Precipitating antitoxin will fix complement and cause Arthus and Danysz reactions under the proper laboratory conditions [3, 1] Occasionally one dose of booster toxoid causes the formation of a high titer of nonprecipitating antitoxin In contrast to precipitating antitoxin nonprecipitating antitoxin fixes complement poorly or not at all and is not able to cause Arthus reactions or Danysz reactions [5, 1] Discrepancies in the *in vivo* (neutraliza tion test) to *in vitro* (precipitin titer) ratio suggest but do not prove the presence of mixtures of precipitating and nonprecipitating antitoxins unless confirmatory assays have been carried out on absorbed sera The *in vivo* to *in vitro* ratio may at times be affected by the concomitant presence in

* The present studies are supported in part by Grant E 1521 from the United States Public Health Service

sera of nonspecific materials (e g rheumatoid factor certain alpha globulins)[14] or by some forms of treatment (e g heat at 56° C)

During the secondary response very high serum antitoxin levels may be associated with antidiphtherial antibodies formed against protein contaminants in toxoid (P proteins) This is possible even when a highly

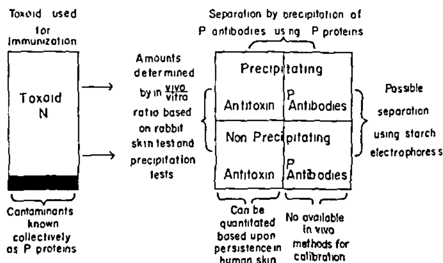


FIGURE 1 Immune responses to diphtheria toxoid

purified toxoid preparation is utilized The multiplicity of antibodies is demonstrable when precipitation tests in a semisolid medium are carried out Quantitative studies indicate that the concomitant presence of precipitating antitoxin and anti P protein antibodies may cause the resultant curve to resemble the flocculation type The configuration of this curve may be altered to the precipitating type when absorption tests are carried out[10] Responses to diphtheria toxoid are depicted in Figure 1

Certain physicochemical properties of antitoxin are (*a*) it migrates electrophoretically as gamma 2 globulin except that in 2 of 40 high titered sera examined antitoxin (nonprecipitating) migrated as gamma 1 globulin[6] (*b*) sedimentation in the ultracentrifuge of precipitating and nonprecipitating antitoxin is similar to the sedimenting properties of the gamma globulin in which they are contained

Antitoxic sera may possess biological properties demonstrable in human passive transfer tests (*a*) persistence at skin sites (*b*) wheal and erythema reactivity (*c*) inhibition of wheal reactions (*d*) irritativeness Factors (*a*) and (*b*) comprise the known attributes of allergic reagin or skin sensitizing antibody I will not employ the latter terms since the phenomena to which they refer can be separately defined and measured using the diphtheria system

Persistence of antitoxin at passive transfer sites can be demonstrated in Schick positive subjects using Schick toxin as challenge antigen Conversion of local skin areas to Schick negative and a subsequent reversion to Schick positive is indicated by the disappearance and reappearance of toxic reactions in the presence of appropriate controls[7] (Figure)

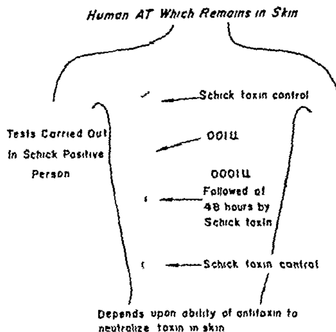


FIGURE 2 Human skin test Toxin neutralization test
Antitoxin in an amount as small as .0001 unit (combined or uncombined with toxoid) at a passive transfer site may be associated with wheal formation following challenge with toxoid If a serum contained only this small amount as wheal reactive antitoxin in addition to wheal unreactive precipitins it would not really be possible to determine its identity as precipitating or nonprecipitating antitoxin The problems in attempting to correlate wheal activity with other forms of immunological measurement are obviously very difficult

We find that length of persistence in skin is related to the *amount* of antitoxin injected whether precipitating or nonprecipitating antitoxin is used and whether or not antitoxin is associated with wheal reactivity Antitoxin equivalent to Schick toxin persists in skin for much less than one week but larger amounts of antitoxin persist for longer periods of time If antitoxin is associated with wheal reactivity the wheal property disappears concomitant with reversion from the passively induced Schick negative state to Schick positivity This finding is correlated with previous studies of immediate reactions to the Schick test Marked immediate wheal

reactions were present in Schick negative persons but not in Schick positive persons

The wheal property may be associated with precipitating and non precipitating antitoxins—a confirmed finding which supplements an earlier observation that wheals were caused only by nonprecipitating antitoxin [11] Among sera containing both antitoxin and the wheal property, electrophoretic separation into wheal poor antitoxic and nonantitoxic fractions may be carried out Recombination of these fractions again provides a wheal rich component Similar experiments using precipitation with toxin as a fractionation method have been less successful In these experiments carried out using isotope (I^{131}) labeled toxin it has not been possible to exclude the presence in supernates of biologically reactive combination products The findings following electrophoresis suggest that antitoxin and some other nonantibody material are needed in order to cause wheals in the presence of toxoid This is of interest in view of previous findings that wheal rich antitoxins were readily converted to wheal poor antitoxins by (*a*) heat at 56° C (*b*) cold ethanol serum fractionation (*c*) exposure to certain conditions of pH or ionic strength [9]

Wheal reactivity to toxoid does not occur during primary immunization A single booster dose of toxoid does not cause wheal reactivity as often as the same amount of toxoid which is given in multiple frequently spaced doses Marked reactions occur most often following very frequent small intracutaneous booster doses of toxoid Treated allergic persons who remain symptomatic despite treatment are also likely to possess immediate reactivity to toxoid

Many individuals with rheumatoid arthritis or rheumatic fever and occasionally normal persons possess sera which cause irritative reactions immediately following passive transfer into skins of normal recipients This property does not appear to be related to prior immunization procedures When present it may be a problem related to studies of blocking or inhibiting antitoxic sera because the inhibition test depends upon interaction (in mixture) of antitoxin with toxoid so that toxoid becomes unavailable for combination with wheal causing antitoxin at prepared sites Blocking antitoxic sera which are also irritative would cause skin reactions regardless of the extent to which toxoid was bound [8]

I believe the primary advantage of the diphtheria system in studying immediate wheal allergies resides in the availability of a neutralization test to provide information about one antibody—that is antitoxin—in human skin The heterogeneity of responses observed in humans following immunization with toxoid would at least depend upon (*a*) status of the host (*b*) manner in which immunization is carried out (*c*) tests employed to measure immune responses We can feel certain that neutralization tests measure only antitoxin Tests which measure wheal reactivity may calls

brate something more than antitoxin alone Further investigations should be based upon comparisons in different tests of a single antibody with the whole serum from which it was derived

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WILLIAM H TALIAFERRO (Chicago Illinois) I wish to discuss the role of avidity in the heterogeneity of hemolysins Avidity is one of the chief characteristics which diversify antibodies of identical immunological specificity It affects the primary union and equilibrium of the first stage reaction The degree to which it affects the secondary manifestation of the antigen antibody reaction depends upon the varying occurrence of immunologically nonspecific factors such as Jerne [2] has discussed in relation to the speed of flocculation of diphtheria antitoxin The effect of avidity when it does modify the secondary manifestation is directly opposed in those cases such as precipitation and agglutination in which the secondary reaction removes antibody from the solution of reactants and in those cases such as lysis in which antibody is liberated to continue the reaction In both cases a high avidity would be expected to be associated with low rates of dissociation and high reaction velocities These characteristics result in an efficient precipitin system but in a very inefficient hemolysin activity

It is generally believed that during hemolysis the antibody forms a complex with the cell and in the presence of complement induces hemolysis Recently Weinrach and Talmage [6] suggested that there is an optimum avidity resulting in an optimal turnover time for antibody during hemolysis Below the optimal avidity the time would be so short that the cell antibody complex would be too short lived to fix complement and induce hemolysis Above the optimum the cell antibody complex would dissociate so slowly that only a relatively few lytic injuries would be produced

Bowman *et al* [1] showed that complement is destroyed during lysis but that antibody can dissociate after lysis and initiate new lytic injuries provided sufficient complement is present They also first demonstrated the transfer of hemolysin from both stromata and red cells to other red cells Intersite transfer on the same cell is undoubtedly more important than intercellular transfer but the latter probably gives a relative measure of the former

We [4] have developed a measure of intercellular transfer based on the work of Weinrach *et al* [5] Radiochromate ($Na_2Cr^{51}O_4$) is used to label red cells because Cr^{51} specifically labels hemoglobin and thus makes it possible to determine the hemoglobin derived from chromium labeled cells The actual measurement in 50 per cent hemolytic units of intercellular transfer is illustrated in Figure 1 One half a unit of washed normal sheep red cells

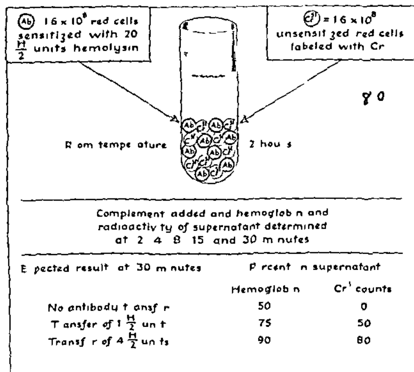


Figure 1 Schematic representation of the intercellular transfer in terms of 50 per cent hemolytic units from unlabeled sensitized to Cr labeled unsensitized sheep red cells during 2 hours *in vitro* contact at 22 C (From a Rice Institute pamphlet see reference 3)

(1 6 × 10) is sensitized with twenty 50 per cent hemolytic units of a rabbit Forssman hemolysin The sensitized unlabeled cells are put in contact with an equal number of unsensitized Cr^{51} labeled cells for hours at room temperature During this interval intercellular transfer of antibody can take place from sensitized unlabeled to unsensitized Cr^{51} labeled cells The mixture is then incubated at 37 C for various times up to 30 minutes with excess complement and hemolysis is stopped abruptly with citrate In the supernatant total hemoglobin is determined photometrically and Cr^{51} labeled hemoglobin is determined by gamma counts

The procedure and three possible results are given in Figure 1 Results from the first antiserum in the figure indicate that no antibody was transferred because 50 per cent of the total cells were hemolyzed and no Cr was found in the hemoglobin of the supernatant Since the sensitized cells were not saturated with antibody, hemolysin which leaves them could attach itself to sensitized or to Cr labeled cells at random Therefore the lysing of half of the Cr^{51} labeled cells indicates the transfer of a full

50 per cent unit of hemolysin Thus for the second antiserum in the figure one 50 per cent hemolytic unit was transferred because 75 per cent of the total cells were hemolyzed and 50 per cent of the total Cr^{51} was in the hemoglobin of the supernatant For the titration of a given antiserum varying amounts of hemolysin are used to sensitize the red cells and are treated as above with incubation with complement for the 30-minute interval From 20 to 100 hemolytic units are used for avid serums and from 0 5 to 20 units are used for nonavid serums In a study of various serums we have found that 5 or more 50 per cent hemolytic units per 20 units used for sensitization transfer in nonavid serums from normal rabbits (which contain low titered natural antibody) or from rabbits within 4 days after a single injection of heated sheep red cell stromata In marked contrast less than one half of a hemolytic unit per 20 units used for sensitization transfers in avid serums at peak titer during initial and anamnestic responses and for several weeks thereafter The great increase in avidity which takes place shortly after 4 days following a single injection of antigen must chiefly involve the large hemolytic antibody since the small antibody appears later It is thus evident that a given amount of nonavid hemolysin is more efficient hemolytically than the same amount of avid hemolysin under the conditions of our titration

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GENERAL DISCUSSION

CHAIRMAN CHASE I am left somewhat confused Perhaps it would be helpful for the group to see that last slide of Dr Karush s and to ask him whether he used more than one sample of immune serum and to develop in greater detail the evidence of heterogeneity in his system Will Dr Karush amplify his remarks at this time

DR KARUSH With respect to the serum which is employed perhaps I should have mentioned that these were purified preparations made from pools of sera obtained from seven to ten rabbits after one course of injections so the heterogeneity which is found here represents a heterogeneity to which contributions have been made by at least seven and at most ten rabbits Whether the heterogeneity of individual rabbits will be any different I think perhaps Dr Pressman can answer

CHAIRMAN CHASE May I ask whether you find this constant of irrespective of the avidity of the serum?

DR KARUSH Within a certain range We have examined approximately for the DIP system six preparations of purified antibody Their average constants have varied over a five or six fold range Each of these preparations has given an extrapolated value for the binding namely the antibody valence of o to within 5 per cent

CHAIRMAN CHASE Thank you for this clarification Now may we see your final slide?

DR KARUSH It is not directly relevant to the topic It deals with possible ways in which the specificity of antibodies is required We suggest that the formation of antibody involves first the development of a polypeptide the sequence of the amino acids being the same as in normal gamma globulin and then the formation spontaneously of a gamma globulin precursor which is characterized by two things namely that all the sulfurs which subsequently will end up as disulfides are in reduced form as cysteine and that this antibody precursor has the property of binding small molecules nonspecifically

(*Slide* See Figure 5 of Dr Karush s paper, p .6) The gamma globulin precursor is entirely hypothetical at the present time It is represented in the reduced form in the sense that all the disulfides – there are twenty to twenty five disulfides in the gamma globulin molecule – are in the SH form This molecule is assumed to have the property to combine nonspecifically with antigenic groupings analogous to the binding properties exhibited by serum albumins We assume that during the transient lifetime of this complex formed by precursor and antigenic group there is oxidation of the SH s to the disulfides By virtue of the disulfide formation the precursor becomes antibody When this takes place in the absence of the antigenic group this precursor becomes a nonantibody gamma globulin

CHAIRMAN CHASE With regard to Dr Raynaud s exceedingly interesting

work the important point that he has presented deserves recounting — that the pure toxin molecule in his estimation is one that directs antibodies to the toxic groupings present on it but at other times to other parts of the same molecule so that there are different areas on the toxin molecule that are capable of directing the formation of antibodies Antibodies to the nontoxin groupings of the molecule do not neutralize toxin but combine specifically with it the ratio between these two classes of antibodies — those that are directed against the prosthetic toxic groupings *versus* those that are directed against the nontoxic part of the molecule — may well control avidity and explain heterogeneity

CURTIS A WILLIAMS JR (New York New York) I should like to address a question to Dr Raynaud and also make a comment

First the question Has this serum from the horse where you found antibodies in the alpha-2 region been controlled in the immunoelectrophoretic examination with an antiserum to horse serum so that one might see whether or not the actual immunochemical gamma globulin or beta globulin is migrating at a higher mobility perhaps due to complex with other proteins

I found in earlier studies with other horse sera to other antigen systems — and Grabar and I also showed — that these antibody or gamma globulin proteins could complex with other serum components and migrate to higher mobilities with actual concentration maxima of discrete electrophoretic mobility

My comment is that in horse antiserum against complex antigen systems the shift during hyperimmunization from gamma mobility to beta-2 mobility is frequently dependent upon the type of antigen employed Serum albumin toxin and other antigens do manifest this shift to a higher mobility There are some antigens notably in human serum which we studied most extensively the antibodies to which never appeared with a higher mobility even in hyperimmune states There are other antigens still that revealed antibodies of both mobilities as Dr Raynaud showed here in the horse serum of a hyperimmune bleeding

What I should like to emphasize is that antibodies to certain antigens seem never to appear with a *higher mobility* than that of *slow* gamma globulin

DR RAYNAUD First as regards association between the different globulins in sera of the type demonstrated by Williams and Grabar The gamma globulin and the beta antitoxin in my experiments do not represent artificial complexes for we can separate them individually The beta globulin possesses another characteristic it gives a flocculation type curve

Concerning the second question in my opinion the rise from localiza

tion in the gamma to the beta fraction is limited to the protein anti protein system With all of the polysaccharide antigens one obtains only gamma type antibody and correspondingly a precipitating type curve

The unanswered question is why horses exhibit this shift in the protein fraction that contains antibody at the *end* of their immunization Perhaps only horses are subjected to such an intensive immunization It must be remembered that we immunize horses for months and very large amounts of antigen are administered two or three times each week It is true that we do not often induce the type of immunization that shows the shift in antibody from gamma protein to the beta We have immunized rabbits with pure toxoid over a long period of time without encountering antitoxin elsewhere than in the gamma globulin fraction

Berry Campbell (Minneapolis Minnesota) My colleague Dr William E Petersen and I have been driven to a formulation of antigen antibody reactions which agrees with the observations on the heterogeneity of antibody which have been discussed this morning My remarks are apropos particularly to the opening statements of Dr Taliaferro We find a continuum of phenomena from immunity to allergy The left end of this spectrum where immunity dominates rests upon the activity of small monovalent nonprecipitating antibody The right end where allergy predominates rests on the activities of large polyvalent lattice forming antibodies The status of an animal with respect to this spectrum is in part determined by the route of administration of the antigen From the immune end of the spectrum to the allergic end the routes are ordered intravenous subcutaneous intramuscular to intradermal With regard acute infectious disease immunity extends along the entire spectrum [illegible] allergic disease the left end relates to desensitization the right [illegible] sensitization At the immune end of the spectrum transplants of [illegible] show acceptance at the left end rejection The situation is the [illegible] cancer transplants At the base of all of these phenomena lie the [illegible] characteristics of the different types of antibody

II

Detection of Antibodies in Human Sera

Chairman BRAM ROSE, M.D. (*Montreal, Canada*)

4

*The Detection and Nature of Nonprecipitating Antibodies in Allergic Sera**

ALEC H. SEHON, Ph.D.
(Montreal, Canada)

The most striking feature of the skin sensitizing antibody is that it has the ability to sensitize normal skin and remains attached to the site of injection for prolonged periods [4, 35] Its ability to sensitize skin is however, lost on heating at 56 C for periods of 1 to 4 hours [33] As far as this author is aware no evidence has been presented that skin sensitizing antibodies can combine *in vitro* with appropriate allergens On the other hand blocking antibodies have been suspected for a long time to be able to combine with the allergen(s) *in vitro* on account of their ability to inactivate the latter [24, 30] Results obtained in this laboratory, [19, 20, 38] which will be reported here, indicate that a firm combination does also occur between the reaginic factor and the allergen

In terms of the lattice or framework theory [29] precipitating or agglutinating antibodies are considered to be bivalent By contrast the non precipitability of skin sensitizing and blocking antibodies has been attributed [6, 7] to the possibility that both of these antibodies are 'univalent or incomplete and that consequently, sufficiently large insoluble aggregates would not be produced even if combination of these antibodies with the appropriate allergens did occur *in vitro* Theoretically the addition of univalent antibodies to a system containing precipitating antibodies and a common antigen could result in a decrease or increase of the amount of precipitate The actual effect may vary from system to system and would depend on the antigen antibody ratio and on the relative *affinities of the different antibodies for the antigen* A decrease in the amount of precipitate was found by Bukantz Johnson and Hampton [5] when a serum of an individual allergic to ragweed (preincubated with the

* This study from the Division of Immunochemistry and Allergy Research McGill University Clinic Royal Victoria Hospital and the Department of Chemistry McGill University is primarily concerned with antibodies in sera of ragweed sensitive individuals It was supported by grants from the Department of Health and Welfare Canada and the National Institute of Allergy and Infectious Diseases National Institutes of Health U S Public Health Service We are also indebted to the Charles E. Frosst Co Montreal for financial assistance

antigen) was added to a rabbit antiragweed serum Normal human serum by itself did not inhibit the precipitation but remarkably, when it was used along with an allergic serum the extent of the inhibition was greater than with the allergic serum alone In contrast Hampton Johnson Alexander, and Wilson [1] reported an increase in the precipitate for a similar system when ragweed was preincubated with an allergic serum This increase was obtained for various antigen concentrations Unfortunately no quantitative data were given Independently Orlans Rubinstein and Marrack [27] used a grass antigen antibody system with the difference that the allergic serum was decomplemented by an ovalbumin antiovalbumin precipitating system Their results were equivocal as on some occasions an increase and on others an inhibition of the precipitation occurred The differences were always within the limits of experimental accuracy All these results were obtained with sera from both treated and nontreated individuals * and one may conclude that these investigations did not throw any new light on the nature of these antibodies

In the common immunological techniques such as precipitation agglutination complement fixation and/or lysis combination of antibodies with the appropriate antigen(s) leads to a readily visible and measurable product of reaction As will become evident later antibodies in sera of ragweed sensitive individuals are present in exceedingly low concentrations their concentration being about 1000 times smaller than the concentration of antibodies produced in experimental animals In consequence it is not surprising that the products resulting from the combination of these antibodies (which as will be shown are in all probability *divalent) with ragweed constituents are not readily demonstrable in vitro,* and that the application of the standard immunological methods for their detection and measurement has met with little success † As in all chemical reactions the sensitivity and accuracy of any test for antibodies will be enhanced if the mass of antigen antibody complexes can be increased This has been the rationale for the use of antigen coated collodion particles [11 4] or erythrocytes for revealing the presence of antibodies which escape detection by other *in vitro* methods

A number of workers showed that collodion particles or red blood cells coated with the allergen were clumped when suspended in allergic serum In 1941, Cohen and Weller [11] stated in a preliminary report that collodion particles coated with an adsorbed layer of ragweed pollen extract were clumped when suspended in allergic sera The clumping was observed regularly with sera of treated allergic individuals whereas most

* All ragweed sensitive individuals who had undergone at least one series of injections of ragweed pollen extract are referred to as treated Sera obtained from such individuals are referred to as treated sera as compared with nontreated sera from nontreated allergic individuals

† A review of earlier attempts to demonstrate antibodies in allergic sera is given elsewhere [19]

of the sera of nontreated patients gave negative results However, in 1947 a systematic reinvestigation of this technique by Swineford and Houlihan[12] failed to confirm the earlier results These workers were able to obtain clumping of ragweed coated collodion particles only with rabbit antiragweed serum but not with allergic human serum and concluded that the clumping had been due to nonspecific factors Unfortunately it is difficult to assess Cohen and Weller's experiments since they used *only one normal serum as a control throughout their work*

In an attempt to demonstrate antibodies in allergic sera Orlans Rubinstein and Marrack[27] and more recently Feinberg Davison and Flick[14] used Boyden's[2] hemagglutination technique* Grass and ragweed pollen antigens were adsorbed to tannic acid treated red blood cells The former group obtained positive results in only out of 49 cases and then only in relatively low serum dilutions (from 1 6 to 1 96) The results of *the second team are comparable that is only 1 per cent of the untreated* and 67 per cent of the treated sera gave positive results

Suggestive evidence that antibodies present in allergic sera combine with the corresponding allergens has been obtained from complement fixation studies Cavelti[10] showed that complement was frequently bound in the presence of treated sera and that sera of nontreated patients were only rarely capable of complement fixation Furthermore no relation was found between the amount of complement fixed and the titer of the skin sensitizing antibodies in these sera as demonstrated by the Prausnitz Kustner test† Portnoy and Sherman[30] claimed that antibodies in allergic sera could be measured by determining the extent to which these antibodies decreased the amount of complement fixed by a known precipitating system The amount of complement fixed by *a constant quantity* of ragweed and rabbit antiragweed serum was determined in the presence or absence of an allergic serum Inhibition was obtained only by sera containing a high level of blocking antibodies and rarely by sera containing only skin sensitizing antibodies

Another avenue of approach has been explored by Noah and Brand[8] who compared the amounts of histamine liberated *in vitro* from allergic and normal blood in presence of ragweed extract The histamine liberated in the blood of allergic individuals was significantly higher than that in normal blood indicating thus that a specific interaction took place between the ragweed and the antibodies present in allergic serum

Studies in This Laboratory

The present studies were undertaken with a view to developing a simple specific quantitative and sensitive *in vitro* method for the demon

* A modification of this test has been used by Britton and Coombs[4]

† Hereafter referred to as P K test or P K titer

stration of blocking and skin sensitizing antibodies in sera of ragweed sensitive individuals and with a view to elucidating the nature of these nonprecipitating antibodies

*In Vitro Detection of Antibodies in Allergic Sera**

On the assumption that these antibodies were divalent and that their failure to give a visible precipitin reaction was due only to their being present in an exceedingly low concentration † it was felt that the hemagglutination method of Pressman Campbell and Pauling[31] — as also recently used by Stavitsky and Arquilla[41] for the demonstration of antibodies to insulin — could be profitably adapted for our purpose

For this purpose the allergen is coupled to rabbit red cells by chemically stable azo bonds bisdiazotized benzidine being the coupling agent. The sensitized red cells are then suspended in the allergic serum If skin sensitizing and/or blocking antibodies are present specific gelatinous aggregates as shown in Figure 1 are produced In absence of antibodies, the sensitized erythrocytes sediment to the bottom of the test tube to form a compact button which represents a negative pattern This hemagglutination test is simple to perform the main problem being the choice of the correct ratio of bisdiazotized benzidine to antigen for a given amount of red cells However once this is established no further difficulties are encountered and reproducible results are obtained in successive experiments The details of the method are described in the Appendix to this article (p 78)

The sensitivity of the method was established by evaluating it in terms of rabbit antisera containing precipitating antibodies to water soluble ragweed pollen extract (WSR) ‡ bovine serum albumin (BSA) human serum albumin (HSA) and human gamma globulin (HGG) Positive ring tests were obtained with these sera in dilutions only as high as 1 64, whereas hemagglutination titers § for the same sera were 10^4 to 10^7 as shown in Table I Also as can be seen in Table I the immunologic specificity of this reaction was demonstrated by the ability to inhibit hemagglutination by the addition of free soluble antigen Thus addition of a solution of ragweed antigens to a system containing BSA sensitized erythrocytes and anti BSA serum had no effect on the extent of hemag

* This study will be incorporated into the Ph D thesis of Mr J Gordon to be submitted to the Department of Biochemistry McGill University Montreal

† The justification for this assumption is based on results obtained by starch electrophoresis[19, 35] by ultracentrifugation[15] and by removal of antibodies from allergic sera with a ragweed polystyrene conjugate [9]

‡ Prepared according to method described elsewhere[40]

§ Expressed as reciprocal of the highest dilution still giving a positive reaction Usually the samples were evaluated in terms of twofold serial dilutions of the whole serum

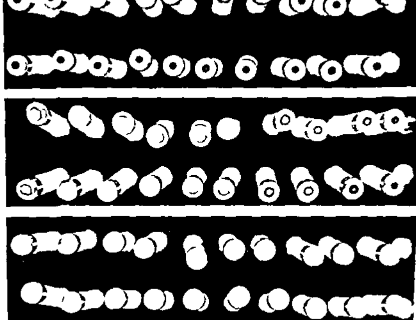


FIGURE 1 Hemagglutination patterns Each row represents tubes [illegible] serum in halving dilutions the concentration of serum decreasing [illegible] to right The top two rows represent a control experiment (in duplica[illegible] normal human serum no hemagglutination is observed in any tube The [illegible] two rows represent an experiment (in duplicate) with allergic serum [illegible] terns represent a gradual decrease in the extent of hemagglutina[illegible] bottom two rows (in duplicate) represent hemagglutination patterns [illegible] with rabbit antiragweed serum The patterns in all ten tubes are p[illegible] actual titer of this serum was 10^7

TABLE I HEMAGGLUTINATION AND INHIBITION EXPERI[illegible]

Antigen Coupled to Red Cells	Inhibitor	Antiserum	Ti[illegible]
WSR	none	anti WSR #1 †	[illegible]
WSR	none	anti WSR #5	[illegible]
WSR	WSR	anti WSR #5	[illegible]
WSR	none	anti BSA	[illegible]
BSA	none	anti BSA	[illegible]
BSA	BSA	anti BSA	[illegible]
BSA	WSR	anti BSA	[illegible]
HSA	none	anti HSA	[illegible]
HGG	none	anti HGG	[illegible]
HGG	HGG	anti HGG	[illegible]
WSR	none	B P	[illegible]
WSR	WSR	B P	[illegible]
WSR	grass pollen	B P	[illegible]
WSR	wormwood pollen	B P	[illegible]

All antisera were produced in rabbits The serum [illegible] from a ragweed sensitive individual

† Repeated intravenous injections of alum precipita[illegible] 2 to 4 months were used for immunization The [illegible] were injected intravenously

TABLE II HEMAGGLUTINATION AND PRAUSNITZ KUSTNER TESTS *

Number of Serum	Donor	Hemagglutination Titer †	P K Titer †
1–22	normal	no reaction	no reaction
SERA FROM INDIVIDUALS SENSITIVE TO OTHER ALLERGENS			
23	sensitive to aspirin	no reaction	no reaction
24	sensitive to grass	no reaction	no reaction
25	sensitive to grass	no reaction	no reaction
SERA FROM RAGWEED * SENSITIVE INDIVIDUALS †			
26	nontreated	64	10
27	nontreated	128	100
28	nontreated	64	500
29	nontreated	128	100
30	nontreated	256	100
31	nontreated	1024	1000
32	nontreated	16	positive ‡
33	nontreated	32	positive ‡
34	nontreated	256	not tested
35	nontreated	64	§
36	nontreated	64	100
37a ‖	nontreated	4	§
37b ‖	treated	128	10
38a ‖	nontreated	32	10
38b ‖	treated	128	§
39a ‖	nontreated	512	250
39b ‖	treated	1024	250
40a ‖	nontreated	128	not tested
40b ‖	treated	512	not tested
41a ‖	nontreated	128	250
41b ‖	treated	2048	500
42	treated	256	1000
43	treated	512	1000
44	treated	32	10
45	treated	128	1000
46	treated	64	100
47	treated	128	500
48	treated	256	100
49	treated	256	100
50	treated	512	100
51	treated	32	§
52	treated	16	§
53	treated	8	§
54	treated	128	500
55	treated	16	100
56	treated	64	100
57	treated	32	100
58	treated	32	100
59	treated	1024	1000
60	treated	2048	1000
61	treated	1024	positive ‡
62	treated	512	positive ‡
63	treated	128	not tested

utination whereas addition of BSA completely inhibited the test Con ersely addition of a solution of WSR to a system containing WSR nsitized erythrocytes and rabbit anti WSR serum or serum from a agweed sensitive individual resulted in complete inhibition On the other and addition of BSA or pollen extracts of other plants to this system id not interfere in any way with the test Reproducible results were btained for any duplicate experiments done with the same serum and he same batch of sensitized erythrocytes as illustrated in Figure 1 lowever using different batches of sensitized erythrocytes the titers aried within a factor of 16 that is ± tubes

The specificity of this method is also borne out by the fact that rag eed coated red cells were agglutinated by each of the 40 sera obtained om a group of 35 nontreated and treated ragweed sensitive individuals id by the 3 sera collected from normal subjects who received a series f ragweed injections No serum obtained from any ragweed sensitive ndividual failed to give a positive hemagglutination reaction On the ther hand the hemagglutination test was negative when applied with agweed sensitized erythrocytes to normal sera (20 sera) or to sera from ndividuals allergic to grass or aspirin All these results are listed in able II together with the hemagglutination and skin sensitizing titers

These results indicate that there exists some parallelism between emagglutination and skin sensitizing titers This may be interpreted in rms of either of the following hypotheses (*a*) the hemagglutination nd skin sensitizing abilities might be due to two distinct properties of ne and the same molecule or (*b*) they are two independent factors ormed concomitantly by nontreated allergic individuals Furthermore would appear that higher hemagglutination titers are given by sera of reated individuals and that the titers obtained with the serum of the ame patient increase on treatment Since formation of blocking antibodies induced on treatment one may suggest that (*a*) the hemagglutination actor is identical with blocking antibody (and that some blocking anti odies are produced even by nontreated individuals in concentrations hich are lower than those required for their detection by the standard *n vivo* test) or (*b*) blocking antibodies produced during desensitization re able to agglutinate sensitized red cells just as other factor(s) found n sera of nontreated individuals

WSR was used for all these experiments

† Expressed as reciprocal of the highest dilution still giving a positive reaction n most cases the samples were evaluated in terms of twofold serial dilutions of the hole serum for hemagglutination and tenfold serial dilutions for P K test

‡ These sera were not diluted for P K tests

§ The P K test was negative for a tenfold dilution of the serum The undiluted erum was not tested

‖ Sera obtained from the same individual before treatment are referred to by etter (a) and after treatment by letter (b)

Characterization of Antibodies in Allergic Sera

To test the above hypotheses and in an attempt to clarify the nature of the hemagglutination factor(s) the following experiments were performed

The skin sensitizing activity of allergic sera of nontreated individuals was destroyed by heating at 56 C — for periods as long as 11 hours — without affecting the hemagglutination titers Similarly the heating of sera of treated allergic individuals or normal immunized subjects did not affect the hemagglutination titers Thus the hemagglutination factor(s) appeared to be thermostable

Sera of treated and nontreated allergic patients were 'absorbed out with an equal volume of packed sensitized erythrocytes In each case the supernatants were shown to have been completely depleted of skin sensitizing and blocking antibodies and of any hemagglutination factor(s) This therefore demonstrates that firm combination had occurred between each of these factors and the ragweed pollen constituents coupled to the erythrocytes To prove that absorption of these factors was an immunologically specific reaction a serum from an individual allergic to both ragweed and grass pollens was absorbed out with ragweed sensitized erythrocytes The supernatant was shown to have been depleted of skin sensitizing and hemagglutination factors with respect to ragweed but to have retained its skin sensitizing ability to grass pollen These results are listed in Table III

At this juncture it is appropriate to present other evidence supporting our view that skin sensitizing and blocking antibodies as well as the hemagglutination factor(s) combine with ragweed pollen *in vitro* Water soluble ragweed antigens were coupled by covalent azo bonds to poly diazotized polyamino polystyrene [9] the resultant antigen polystyrene conjugate being an insoluble polymer * The reactions involved in the preparation of this conjugate are shown in the flowsheet presented in Figure Allergic sera were then exposed to this antigenic adsorbent and it was found that skin sensitizing and blocking antibodies together with hemagglutination factor(s) were completely and specifically removed Heterologous antigens coupled to polystyrene had no absorbing capacity for any of these factors found in sera of ragweed sensitive individuals This method was deemed to provide also the possibility of isolating these factors in a pure and concentrated form subsequent to their absorption by dissociating them from the ragweed polystyrene conjugate Although dissociation of ordinary precipitating antibodies can be readily effected[3] no satisfactory method has so far been devised for recovering the factors present in allergic sera

These experiments will be incorporated into the Ph D thesis of Mr L. Gyenes to be submitted to Department of Biochemistry McGill University Montreal

TABLE III TITERS FOR HEMAGGLUTINATION AND SKIN TESTS AFTER EXPOSURE OF SERA TO ERYTHROCYTES SENSITIZED WITH WSR

Number of serum	Donor	Absorbed with Cells	Hemagglutination Titer	Blocking Titer	P K Titer to WSR
59	sensitive to WSR treated	nonsensitized	1024	not tested	1000
		sensitized	no reaction	not tested	no reaction
31	sensitive to WSR nontreated	nonsensitized	1024	no reaction	1000
		sensitized	10	not tested	10
		sensitized 2nd time	no reaction	not tested	no reaction
60	sensitive to WSR treated	nonsensitized	2048	32	1000
		sensitized	10 †	no reaction	10
0	nonallergic volunteer immunized with ragweed	nonsensitized	8192	64	no reaction
		sensitized	no reaction	no reaction	no reaction
66	sensitive to WSR and to grass	nonsensitized	512	not tested	500 ‡
		sensitized	no reaction	not tested	no reaction ‡

Expressed as reciprocal of the highest dilution still giving a positive reaction

† This serum was not absorbed out with a sufficient amount of sensitized erythrocytes

‡ An identical P K titer of 500 to grass extract was obtained with this serum before and after exposure to WSR sensitized erythrocytes

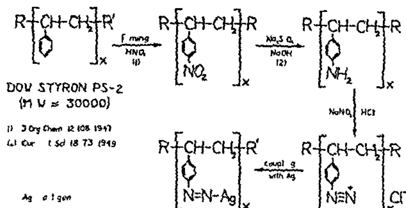


FIGURE 2 Flowsheet of the preparation of polystyrene antigen conjugates

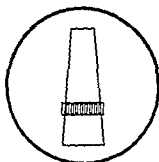
Next we turned to a number of fractionation procedures in the hope that these various factors could be separated from each other As was reported in earlier studies [3, 8 ... 38] skin sensitizing and blocking antibodies were shown to migrate electrophoretically as gamma 1 and gamma globulins, respectively * Therefore if hemagglutination was due to blocking antibodies only one would expect to find the hemagglutination factor(s) primarily in the gamma globulin region A number of allergic sera were fractionated by starch and continuous paper electrophoresis (Spinco CP Apparatus) Hemagglutination ability was found to be associated with all serum subfractions throughout the gamma 1 and gamma -globulin region with which skin sensitizing ability was also associated Similarly applying the chromatographic fractionation procedure on cellulose as used by Humphrey and Porter it was shown that skin sensitizing antibodies and the hemagglutination principle(s) were always associated with each other Thus both zone electrophoresis and chromatography failed to divorce these factors from each other

Allergic sera on prolonged dialysis in standard Visking tubing did not lose their skin sensitizing and hemagglutination ability and these factors were not separated by ultrafiltration from the rest of the serum proteins It was therefore supposed that these factors were associated with serum components of a molecular weight higher than about 30 000 Finally fractionation of the factors in question was attempted in the ultracentrifuge (Spinco Model E Apparatus) in preparative cells provided with both fixed [45] and moving [4] partitions (Figure 3) These cells were essentially identical but we feel that the cell of Yphantis and Waugh is to be preferred since disturbances due to convection were not observed with it As is commonly known normal (and allergic) sera [38, 46] are resolved in the ultracentrifuge into two major peaks with sedimentation constants of the order of 4 5 and 6 5S and a minor peak of heavier material – amounting to about 3 per cent of the total protein – with a sedimentation constant of about 18 to oS The sedimentation patterns were observed with the standard Schlieren cylindrical lens system and permanent records were obtained on photographic plates A typical sedimentation pattern in the moving partition cell is shown in Figure 4 In most experiments the sera were diluted with physiological saline prior to centrifugation

In these experiments the peak corresponding to the heavy serum component(s) was allowed to sediment from the upper into the lower compartment As soon as it had crossed the partition the rotor was stopped and the serum fractions were withdrawn from each compartment

* However Cann and Loveless [9] consider also that skin sensitizing antibodies reside in beta globulins

FIXED PARTITION CELL



MOVING PARTITION CELL

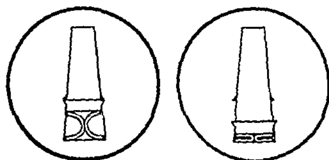


Figure 3 Schematic representation of the fixed and moving partition cells The lower left picture represents the cell at rest and the lower right picture represents the cell during centrifugation



Figure 4 Ultracentrifugal pattern of allergic serum in the moving partition cell The first and the last frames were photographed at 5000 r p m the second third and fourth frames were photographed at 19 26 and 31 minutes respectively after the rotor attained full speed

for determination of skin sensitizing blocking and hemagglutination titers In a preliminary experiment centrifugation was continued until all three peaks had migrated into the bottom compartment The top compartment was found to be devoid of any of these factors and this is considered to be an independent confirmation that these factors are associated with materials with molecular weight higher than that of serum albumin In another experiment performed in the cell with the fixed partition the lower compartment was filled with normal serum and the upper compartment with a serum from a nontreated allergic individual Centrifugation was stopped when the heavy component (18 to 20S) had migrated into the lower compartment Both skin sensitizing and hemagglutination abilities were associated only with the bottom fraction This was a clear indication that both skin sensitizing antibodies and the hemagglutination factor(s) migrated with the heavier component

In all other experiments listed in Table IV the cell was filled throughout with the same serum As can be seen from Table IV with allergic sera containing no blocking antibodies skin sensitizing antibodies and the hemagglutination factor(s) were always found together in the lower compartment With heat inactivated sera no skin sensitizing antibody was found in either of the compartments however the hemagglutination factor persisted in the lower compartment With sera of treated allergic individuals it was shown that while the skin sensitizing antibody migrated into the bottom compartment the hemagglutination factor(s) and blocking antibodies were found in both compartments This suggests therefore that in treated sera the hemagglutination factor is due to a different molecule from the one which is responsible for hemagglutination with nontreated serum and that this factor as well as blocking antibodies is associated with the 6 5S component To confirm unequivocally this conclusion the sera from two immunized nonallergic volunteers devoid of skin sensitizing antibodies were separated by centrifugation In both cases the hemagglutination factor was found in the two compartments Thus it is fairly safe to conclude that during desensitization a hemagglutination factor with distinct molecular properties is produced that is its sedimentation constant is about 6 5S whereas that in nontreated *allergic sera has a sedimentation constant of 16 5S* *

Mechanism of the Hemagglutination Reaction

The experiments with precipitating antibodies suggest that in principle the mechanism of the hemagglutination reaction is similar to that of the precipitin reaction The agglutination of sensitized red cells may be con

* Since sedimentation constants were not obtained for different concentrations extrapolation of these values to zero concentration was not possible The sedimentation constants determined for the heavy component were approximately 16 5S and one would expect these values to be even larger at infinite dilution

TABLE IV HEMAGGLUTINATION AND P K TITERS* OF SERUM FRACTIONS OBTAINED BY ULTRACENTRIFUGATION

Serum	Fraction	Hemagglutination Titer	P K Titer
1 S †	top bottom	2 64	1 100
1 S	top bottom	not tested not tested	no reaction 1000
1 S (heated)	top bottom	no reaction 32	no reaction no reaction
2 S	top bottom	§ 64	§ 1
3 B ‡	top bottom	50 200	no reaction no reaction
4 B ‡	top bottom	64 256	no reaction no reaction
5 S B §	top bottom	200 100	no reaction 100
5 S B (heated)	top bottom	2000 56	no reaction no reaction

* Titers are expressed as the reciprocal of the highest dilution still giving a positive reaction These titers are only relative No comparison with the original undiluted whole serum is made S represents sera containing skin sensitizing antibodies B represents sera containing blocking antibodies

† Similar results were obtained in five experiments done with this serum

‡ These are sera obtained from normal volunteers immunized with ragweed

§ Blocking titers were 16 and 32 for the top and bottom fractions respectively

sidered to be due to their being cross linked into a three dimensional gel by divalent antibody molecules The inhibition of the agglutination with free soluble antigen lends further support to this belief To prove unambiguously this claim one would have to succeed in inhibiting this reaction with antibody in excess and to show that the reaction would not occur with truly univalent antibody Unfortunately inhibition in antibody excess does not occur readily even in ordinary precipitin reactions * and up to the time of writing the present paper we have not been able to produce univalent antibody † However we have obtained further evi

* This prozone effect has been recently studied in detail by Nisonoff and Winkler[25]

† We intend to prepare univalent antibodies by enzymatic degradation of precipitating antibodies If the view presented here is correct these preparations should inhibit both the precipitin and hemagglutination reactions and should still be capable of neutralizing the antigen

dence for the similarity between the hemagglutination and precipitin reactions by reversing the experimental conditions — that is by coupling precipitating antibodies to red cells and adding the antigen in varying amounts to a series of tubes containing a constant amount of antibody coated red cells The results of these experiments illustrated in Figure 5

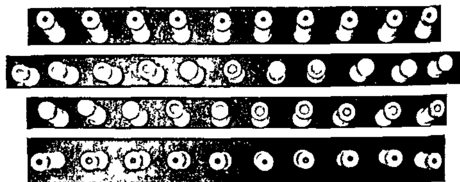


FIGURE 5 Hemagglutination patterns The first and fourth rows represent controls The tubes in the first row contain cells sensitized with rabbit antiragweed antibodies in diluent The tubes in the fourth row contain non sensitized cells in ragweed solution In the second and third rows positive reactions have been obtained with cells sensitized with rabbit antiragweed antibodies The antigen (WSR) was in solution and was added in serial halving dilutions (the maximum concentration was in the outermost tube in the second row)

closely resemble the patterns of Figure 1 and the gradation of the resulting patterns follows at least qualitatively the characteristics of a precipitin curve that is the extent of aggregation increases at first with increasing amounts of antigen (antibody excess region), reaches a maximum (optimum zone) and finally decreases in excess antigen * In consequence we feel inclined to consider the hemagglutination reaction obtained with allergic sera as being due to divalent antibodies † and that — as assumed at the very beginning — these factors have previously escaped detection by ordinary immunological methods on account of their low concentration The plausibility of this assumption is borne out by the ultracentrifugal studies All the serum components which have a sedimentation constant of about 18S represent only about 3 per cent of the total serum proteins and one could therefore safely predict that skin sensitizing

In these experiments only gamma globulin fractions of rabbit antisera were used These experiments could be performed only with extremely rich antibody preparations since presumably with weaker preparations the red cells were coated primarily with nonantibody globulins Experiments are under way to isolate pure" and enriched antibody preparations by fractionation of antisera on homologous antigen polystyrene conjugates

† We consider that the valency of antibodies must be at least 2

antibodies represent only a small fraction of these heavy components As a matter of fact preliminary quantitative ultracentrifugal results indicate that the amount of material which is removed from the heavy component by absorption of allergic sera with a ragweed polystyrene conjugate is negligible

Comparison of hemagglutination titers with the titers obtained by ring and/or Oudin tests for precipitating antibodies offers an indirect but plausible proof for this claim As shown in Table I hemagglutination titers ranged from 10^1 to 10 while the titers of the same sera by ring or Oudin tests were not higher than 64 or 1 8 One may therefore, conclude that the sensitivity of the hemagglutination test is about 10^3 to 10^5 higher With allergic sera the highest hemagglutination titers were of the order of 10 to 10^3—that is about 10 to 10^4 lower than the titers obtained with antisera produced in experimental animals Assuming then that the nature of the hemagglutination reaction with both allergic and animal sera is the same one could hardly expect to detect any visible aggregation on addition of soluble allergens to allergic serum As mentioned in the introductory part of this paper a number of investigators [4-7] using Boyden s technique have demonstrated antibodies in sera of ragweed sensitive individuals with varying degrees of success Although no doubt this technique is highly versatile and sensitive we feel that its main draw back is that the antigens are attached to red cells only by relatively weak adsorption forces and not by stable covalent bonds The mechanism of the hemagglutination reaction is probably identical in the Boyden technique and in the technique described in this paper However since in our method ragweed antigens were coupled by azo bonds no antigens could have dissociated spontaneously from the sensitized erythrocytes as was shown to be the case with Boyden s technique [1] This is the main reason in my opinion for the higher sensitivity achieved in the studies presented here in comparison with previous investigations [24, 25] since any desorption of antigen would lead to partial or complete inhibition of the test and to a poor reproducibility of the results This may also be in part the reason for the findings of Feinberg *et al* [14] that complete desensitization of a site of normal skin sensitized with allergic serum could not be achieved with tanned stromata coated with ragweed components These complications are probably minimized when the antigens used for coating tanned erythrocytes are large molecular weight proteins since desorption will be then more difficult on account of the greater number of links between the protein and red cells

For the hemagglutination techniques the sensitized red blood cells are necessary only to provide the bulk which through cross linking with antibodies will lead to visible aggregation For the full understanding of the mechanism of hemagglutination reaction it is essential to establish

the minimum number of antibody molecules per sensitized erythrocyte which will still lead to agglutination So far, preliminary experiments indicate that 10 to 100 antibody molecules per sensitized red cell are sufficient to give a discernible titer * In the hemagglutionation experiments with rabbit antibodies to ragweed coupled to erythrocytes as little as 10^{-7} mg of ragweed protein yields a visible reaction In comparison the sensitivity of the agar gel technique is lower that is it is claimed [8] that this technique will detect 10^{-3} to 10^{-4} mg of antigen

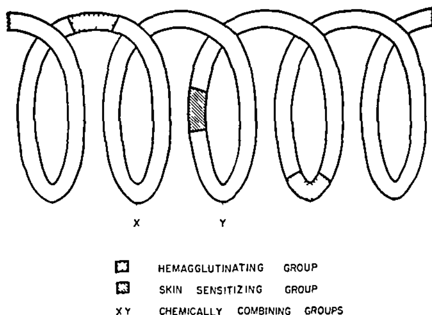


FIGURE 6 Configuration of a hypothetical molecule endowed with hemagglutination and skin sensitizing characteristics

The fact that skin sensitizing activity may be destroyed by heating without impairing the hemagglutinating ability may lead one to believe that in nontreated sera these are two independent properties associated with two distinct types of molecules Nevertheless the possibility that both these factors are associated with one and the same molecule cannot be excluded on the basis of the results of the various fractionation procedures This supposition can be justified in terms of a hypothetical protein molecule containing both these factors as illustrated in Figure 6 Thus the two dotted regions would represent portions of the molecule which are sterically complementary with respect to the antigenic group-

* Quantitative experiments with I^{131} labeled antibodies and antigens are under way to establish this more definitely

ings in ragweed and the lined portion would represent the grouping which has the ability to become fixed to skin and tissues that is the part of the molecule mediating skin sensitization The groups X and Y would represent chemically reactive portions such as -SH groups which on heating may condense to lead to an irreversible change in the configuration of the protein molecule Thus the grouping responsible for skin sensitization without necessarily being destroyed during heating (although this may also occur) may become completely shielded that is its attachment to the skin may be blocked and thus its ability to sensitize the skin may be inhibited This change however may occur without affecting the ability of the other two groupings to combine with ragweed allergens which are coupled to erythrocytes or to polystyrene

Similarly in view of our inability to separate blocking activity from hemagglutinating capacity *in the same fractions of treated serum* one may suggest that as with skin sensitizing antibodies these two different attributes are different manifestations of one and the same molecule and that blocking antibodies may be regarded as normal antibodies produced in response to immunization To substantiate this proposal we have shown that antisera to ragweed produced in rabbits goats and dogs not only had the ability to hemagglutinate sensitized red cells but could also block the reaction of ragweed allergens in human skin sensitized with reagin containing serum Hence one may consider that blocking activity is the result of combination between antibodies and ragweed and that the latter is thus inactivated In this paper hemagglutination is considered to be due to combination of these antibodies with ragweed constituents coupled to erythrocytes Since hemagglutination is still exhibited by nontreated allergic sera even after heat inactivation of their skin sensitizing ability one would expect that these sera should possess also blocking activity * The fact that no blocking activity has ever been reported to exist in non treated sera (after heating) may be due to its being present below the threshold level required for its detection by the *in vivo* test This interpretation is offered on the basis of the following experimental results Three normal individuals were immunized with ragweed None of the sera contained skin sensitizing antibodies However hemagglutination activity was found in all three sera in varying titers in dilutions of 64 000 2048 and 1 8 Blocking activity could be demonstrated by the *in vivo* test only in the serum with the highest hemagglutination titer (hemagglutination titer = 64 000 blocking titer = 64) The gamma globulin fractions of the other two sera were then concentrated fivefold and on retesting these

* This effect should be exhibited irrespective of whether the groupings responsible for hemagglutination and skin-sensitizing activities reside on the same or on different molecules

concentrated fractions blocking activity * was indeed demonstrated in both cases and the hemagglutination titers had also increased †

On the other hand if no blocking activity is revealed by nontreated sera after heating then one would be inclined to assume that the groupings responsible for hemagglutination ability of the skin sensitizing antibody on the one hand and of the blocking antibody on the other, are complementary to different portions of the ragweed molecule(s) This is in agreement with the findings [1, 47] that ragweed contains a multiplicity of antigens Furthermore two independent investigations done in this laboratory prove the diversity of antibodies to ragweed found in allergic sera It was shown that (*a*) there are at least two reagins in sera of nontreated allergic individuals directed against different antigenic constituents in ragweed [34] and (*b*) during desensitization a new type of reagin(s) may be produced to a dialyzable ragweed fraction to which apparently no skin sensitizing antibodies are produced spontaneously by nontreated allergic individuals [35] This, of course is not surprising, considering the chemical complexity of ragweed pollen and the somewhat similar findings of Kuhns [3] who has shown that three types of antibody to diphtheria toxoid may be produced The diversity of antibodies to ragweed allergens with regard to their sedimentation constants would indicate that different mechanisms of antibody formation must be responsible for their production A similar suggestion has been made in review articles by Taliaferro [43] and by Talmage [44] for Forssman antisheep red cell antibodies produced in rabbits which have been found both with the low and the high molecular weight serum components As regards antibodies found in the heavier globulin fraction, one may also cite Rh antibodies,[7] Wassermann antibodies [13] factors present in sera of patients with rheumatoid arthritis which may be involved in the sheep cell agglutination reaction [17] and auto antibodies to thyroid [32]

APPENDIX

The essential steps of the hemagglutination method are (*a*) coupling of the antigen to rabbit red cells through chemically stable covalent azo bonds and (*b*) clumping of these sensitized red cells by homologous antibody

* Since blocking antibodies are produced during immunization and since they have characteristics similar to those of gamma globulins (i e electrophoretic mobility and sedimentation constant) it is tempting to consider blocking antibodies as normal antibodies (usually associated with gamma globulins) In this connection it is appropriate to mention the recent results of Follensby and Lowell [16] who have shown that combination occurs between a component of ragweed pollen and the blocking antibody when the latter is present in a specific precipitate obtained with rabbit anti human gamma globulin serum Although the similarity between blocking antibodies and normal globulins was not explicitly brought out by these workers this conclusion is obviously implied in their results Thus blocking antibody would appear to possess also antigenically similar properties to those of normal human globulins

† Nontreated sera from ragweed sensitive individuals are being tested for blocking activity by the same procedure

Sensitization of Red Cells

Bisdiazotized benzidine (BDB) was used to couple the antigen to red cells It was prepared by dissolving 0 23 g of benzidine in 45 ml of 0 N hydrochloric acid and adding 0 175 Gm of sodium nitrite in 5 ml of distilled water to this solution at 0 C The reaction was allowed to proceed for 30 minutes with intermittent stirring Aliquots of the solution were then placed into ml vials quick frozen at −78 C in a dry ice acetone bath and stored at −20 C until required Some of the batches were used over periods as long as 5 months without detectable deterioration For each experiment the content of a vial immediately after thawing was diluted fifteenfold with 0 15M phosphate buffer at pH 7 3 and was used for coupling the antigen to the red cells

Blood was collected from the marginal ear vein of a rabbit into an equal volume of Alsever s solution and stored at 4 C for as long as 10 days The red blood cells when needed were separated by centrifugation and were washed 3 times with chilled saline

Normal rabbit serum (NRS) was collected from the same rabbit which supplied the erythrocytes The serum was heated at 56 C for 30 minutes to deactivate the complement and was stored at 4 C for 7 to 10 days The diluent solution consisted of NRS diluted hundredfold with phosphate buffer

For sensitization of red cells † the ratio of benzidine to antigen was found to be critical This ratio was established for each batch of BDB (*a*) by varying the BDB concentration and keeping the antigen concentration constant and (*b*) by varying the antigen concentration and keeping the BDB concentration constant Each batch of sensitized cells was tested with a ragweed sensitive serum and with a normal human serum The concentrations of benzidine and ragweed adopted were the ones which gave the highest sensitivity — that is the highest titer with the allergic serum and no reaction with the normal human serum

In the present experiments 3 ml of 75 mg per cent WSR solution were mixed with 0 1 ml of a 50 per cent suspension of washed red blood cells and 0 5 ml of the BDB phosphate was added to this suspension ‡ The reaction mixture was kept at room temperature for 15 minutes with occasional stirring It was then centrifuged at 500 g at 4 C for 5 minutes the supernatant was discarded and the cells were washed with 3 5 ml of the diluent solution (the wash was always colorless) The cells were then redispersed in 2 5 ml of diluent

The Hemagglutination Test

In order to remove nonspecific agglutinins all sera before testing for antibodies were absorbed out at room temperature for 20 minutes with an equal volume of packed washed nonsensitized cells The cells used for the

The buffer used in all experiments was prepared by mixing 15 ml of 0 15M Na_2HPO_4 with 49 ml of 0 15M KH_2PO_4

† The cells have to be sensitized for each experiment

‡ The BDB phosphate solution was prepared by diluting BDB fifteenfold with the phosphate buffer

absorption and those for the sensitization were always from the same rabbit. Subsequently twofold serial dilutions of the serum with the diluent were prepared. The volume in each tube was 0.5 ml.* For inhibition studies the antigen was added at this stage in a volume of 0.1 ml. (the concentration was arbitrarily chosen as 3 mg./ml.). Finally 0.05 ml. of the sensitized cell suspension was dispersed in each tube. For each experiment two controls were used: (*a*) in one series of tubes the serum was replaced by the diluent; (*b*) in another series untreated cells were used instead of sensitized cells. Most experiments were done in duplicate. The tubes were allowed to stand overnight at room temperature to permit the development of specific patterns which once formed remained unchanged for days. For inhibition tests the antigen could be added also after the positive patterns were obtained. The original supernatant was removed and the inhibitor—that is, the antigen—or saline alone was added. The gelatinous patterns were then dislocated by gentle tapping. On reincubation in presence of homologous soluble antigen negative patterns were obtained while addition of saline alone or of a heterologous antigen did not interfere with the reproduction of the positive patterns.

SUMMARY

The methods used for the *in vitro* detection of antibodies in sera of ragweed sensitive individuals are reviewed briefly.

A new hemagglutination method for the demonstration of these antibodies is described. The essential steps of this method are: (*a*) coupling of ragweed constituents to rabbit red cells by stable covalent azo bonds and (*b*) aggregation of these sensitized erythrocytes by antibodies in allergic sera. The gelatinous patterns obtained are believed to be due to cross linking of sensitized erythrocytes by divalent antibodies into a three dimensional network. It is suggested, therefore, that there is no reason to consider that antibodies in sera of ragweed sensitive individuals are univalent or incomplete.

These sera were fractionated by ultracentrifugation in partition cells. The results of these experiments demonstrate that skin sensitizing and hemagglutination abilities in sera of nontreated allergic individuals are associated with the heavy serum components (18S) and that blocking and hemagglutination activities produced during desensitization reside in the normal gamma globulin fraction (6.5S). The possible nature of these entities is discussed.

Skin sensitizing and blocking antibodies have been shown to combine firmly *in vitro* with ragweed constituents.

* The tubes used for this test were 10 by 75 mm. They were suspended through a 1 inch by 10 inches by 1 [illegible] inch sheet of tentest provided with 10 mm. holes and the hemagglutination patterns were observed with the help of a mirror.

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5

*Detection of Antibody Capable of Removing Skin Reactivity in Pollen Extract**

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There is scattered evidence that antibody present as an immunologic precipitate can combine with antigen.[4] We have reported that this principle is applicable to the detection of antibody for ragweed pollen extract.[5]

The procedure we used was as follows. Samples of human serum containing neutralizing antibody for ragweed pollen extract obtained from normal subjects and patients who had received a course of injections of ragweed pollen extract were mixed with a potent rabbit antiserum for human gamma globulin in approximately optimal proportions and the resulting precipitates were carefully washed in chilled saline. The precipitates were then thoroughly dispersed in diluted pollen extract and removed by centrifugation. The supernatants were tested in the skin of patients reacting intensely to ragweed pollen extract by skin test, or in the skin of normal subjects at sites passively sensitized with serum obtained from patients with ragweed pollenosis. If these supernatants exhibited a decrease in skin reactivity as compared with diluted extract unexposed to the precipitates or with the diluted extract exposed to precipitates made with serum from normal subjects who had received no injections of extract, specific binding of antibody with one or more skin reactive components by the precipitate was presumed to have taken place. Only very small traces of human or rabbit protein remained behind in the solution of pollen extract after the precipitates were removed, and these gave no interfering reactions in the skin of the test subjects.

Irregular evidence of binding was obtained at first but it soon became clear that binding was consistently demonstrable when the supernatants

* From the Evans Memorial and Massachusetts Memorial Hospitals, Department of Medicine, Boston University School of Medicine. Supported by United States Public Health Service Grant E 1356(C7).

were tested in the skin of normal individuals at sites sensitized with serum from pollen sensitive patients or in the skin of patients who reacted by skin test to Fraction I * but not to Fraction II [6] This has led to the belief that removal of skin reactivity by the precipitates was mediated by an antibody directed against Fraction I Furthermore since precipitates made with serum containing blocking antibody obtained from injected normal individuals removed skin reactivity it is further believed that antibody other than the skin sensitizing antibody so frequently encountered in the serum of pollen sensitive patients must play a part This antibody is believed to be the neutralizing blocking or heat stable antibody These results would also suggest that the samples of serum used to sensitize the sites in normal skin as just described contained skin sensitizing antibody for Fraction I only If such is commonly the case, titrations in normal skin with serum pollen mixtures containing skin sensitizing antibody as the indicator [1] would measure blocking antibody for Fraction I only This possibility should be taken into account in attempts to correlate levels of blocking antibody as commonly determined with the results of injection therapy with ragweed pollen extract These speculations are made with awareness that the identity of antigenic and skin reactive fractions in ragweed pollen extract are as yet inadequately defined and that the prevailing views concerning blocking and skin sensitizing antibody for pollen extracts may be oversimplifications or even misinterpretations of the available evidence

Preliminary experiments with precipitates made with chicken antibody for rabbit gamma globulin and rabbit antiserum for ovalbumin and ragweed pollen extract have also given clear evidence of binding The experiment with rabbit antiovalbumin confirms earlier work by Follensby and Hooker [5] who used equine antiovalbumin serum and rabbit antiequine serum A patient with ragweed pollenosis was used as the subject for testing the supernatants obtained in the experiment with rabbit anti ragweed serum and again the supernatants contained insufficient rabbit or chicken protein to cause interfering skin reactions

It is tempting to speculate concerning the circumstances under which union between antigen and antibody takes place in the precipitate (Figure 1) Under most conditions the antibody of immediate concern will constitute only a small fraction of the total human gamma globulins present The precipitate will be composed of large numbers of molecules of human gamma globulin possibly other human globulins as well and even larger numbers of molecules of rabbit antibody for human gamma globulin Scattered through the network of interconnecting molecules will be an occasional human gamma globulin molecule specifically modi

* Fraction I was prepared from crude pollen extract by precipitation with ammonium sulfate at 50 per cent saturation Fraction II by precipitation at 100 per cent saturation For details see references 5 and 6

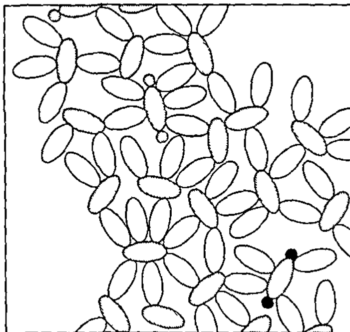


FIGURE 1 Schema representing the composition of an immunologic precipitate obtained when serum from a patient treated with injections of ragweed pollen extract was mixed with rabbit antibody for human gamma globulin The precipitate was then washed and thoroughly dispersed in a solution of ragweed pollen extract The antibody for the skin reactive component in pollen extract is depicted as bivalent although evidence for such an assumption is lacking Open ovals rabbit antibody for human gamma globulin Stippled ovals human gamma globulin Small stippled circles indifferent antigens in ragweed pollen extract (see text) Small solid circles skin reactive antigen (allergen) in ragweed pollen extract

fied to combine with one of the several antigens in ragweed pollen extract Ragweed pollen extract has been shown to contain five or more antigens[1 8] and since the skin reactive (i e allergenic) antigens apparently number only two or three some of the antigens which I shall refer to as the indifferent variety are probably entirely irrelevant so far as measurement of neutralizing antibody is concerned Some or all of these indifferent antigens would presumably give *in vitro* reactions such as precipitation or complement fixation Since injections of pollen extract will serve as a stimulus for the formation of antibody to the indifferent antigens as well as for the formation of blocking antibody *in vitro* tests might give results which at best only paralleled the results of tests designed to measure interference with skin reactivity These circumstances coupled with the lack of precision among the tests with which we are dealing could easily lead to a mistaken belief that *in vitro* tests measured neutralizing antibody itself[4] At the present time therefore

there seem to be insurmountable obstacles to the use of purely *in vitro* procedures in the measurement of neutralizing antibody

These considerations also bear on the distribution of the various types of molecules in the precipitate If the number of indifferent antigens in pollen extract exceeds the number of skin reactive antigens these last may well make up a very small minority of the antigen antibody complexes of which the precipitate is composed If the procedure is to give quantitative information the skin reactive antigen(s) must have the opportunity to combine with all of the antibody for it in the precipitate It will be necessary then that the solution circulate freely through the precipitate and that the sites on the human gamma globulin which can combine with the skin reactive antigens not be encroached upon by molecules of rabbit antibody The extent to which these desirable conditions actually obtain is of course unknown Although optimum conditions can only be determined by experiment one might suppose that the desired reaction would be favored if rabbit antibody were not present in excess if the precipitate were thoroughly broken up in the allergenic solution and if the molecular size of the skin reactive component were small This in all probability has a molecular weight of less than 15 000 a size which must be well below the limiting magnitude since the larger ovalbumin molecule is also readily taken up by the appropriate precipitate

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6

Detection of Human Blocking Antibody to Ragweed by Inhibition of a Complement-Fixation Test

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It has long been known that the sera of patients with allergic diseases of the hay fever and asthma type do not contain precipitins or antibodies demonstrable by the classical *in vitro* methods In general the presence of circulating antibodies in the untreated patient with hay fever has been demonstrated only by the Prausnitz Kustner method of passive sensitization of normal human skin [1]

This reaction is an exceedingly sensitive method of demonstrating allergic sensitizing antibodies and the corresponding antigens but not readily utilized for quantitative determinations The reaction observed is the physiologic response of the capillaries of the skin to histamine (and possibly other intermediaries) released by the union of antigen and antibody Its intensity may be affected by the amount of histamine present in the skin by the physiologic activity of the capillaries and by other factors not readily measured so that the same sample of serum may give quite different end points in different apparently normal skins [2] For this reason absolute end points are of limited value However by its modifications in the dilution and neutralization tests it permits the rough measurement of the relative amounts of antibody in different samples of serum by comparisons made on the same test subject at the same time

Aside from the theoretical difficulties the method of passive transfer involves practical problems in securing an adequate amount of normal skin and in the possibility of transmitting infectious disease especially virus hepatitis For these reasons workers using this biological method have cast jealous eyes on the precise *in vitro* methods of the immunologist and the immunochemist

The presence of the blocking antibody developed as a result of the injection treatment of hay fever was first suspected by Cooke and his co workers [3] on the basis of comparisons of the dilution and neutraliza

tion tests (both made by modifications of the Prausnitz Kustner technique) on sera of hay fever patients before and after treatment More direct evidence of the existence of this antibody was offered by Loveless[8] who showed that heating the serum of the treated patient inactivated the sensitizing antibody without affecting the blocking antibody

The usual methods of demonstrating and measuring the blocking antibody are modifications of Loveless procedure of adding heated serum to mixtures of known amounts of sensitizing serum and antigen in neutralization tests[1] While they depend on comparisons rather than absolute end points they are to some extent subject to the difficulties and errors inherent in the Prausnitz Kustner phenomenon

Since the blocking antibody is believed to manifest its presence by combining with and inactivating the antigen it would seem probable that it might inhibit *in vitro* antigen antibody reactions as well as the Prausnitz Kustner reaction

Hampton Johnson Alexander and Wilson and later Bukantz Johnson and Hampton studied the precipitation of ragweed antigens by rabbit antiserum and showed that the amount of precipitate formed was reduced by the addition of human antiragweed blocking antibody to the system

In hopes of developing a more sensitive *in vitro* method of demonstrating the blocking antibody the authors investigated the method of complement fixation On the basis of our own preliminary studies and those of others [4] of which time does not permit a detailed account it may be stated that in general the sera of pollen sensitive patients untreated or treated do not fix measurable quantities of complement in the presence of the corresponding pollen antigens

The method adopted[9] was based on the observation of Rice[11 1] that certain animal immune sera incapable of directly fixing complement in the presence of antigen nevertheless inhibited the complement fixing properties of serum from another species for the same antigen Rice termed this reaction the indirect complement fixation test and other investigators have utilized it in the study of virus diseases[6] [13 17]

Since the human antiragweed blocking antibody has been shown to lessen the precipitation of ragweed antigen and rabbit antibody it seemed probable that it might also inhibit the fixation of complement by rabbit antiragweed serum

The first step was to establish the characteristics of the fixation of complement by rabbit antiragweed serum Rabbit antisera were prepared by injection of alum precipitated ragweed extract The ragweed antigens used in the complement fixation tests were whole extracts of low ragweed pollen prepared with Coca s alkaline solution and preserved with glycerin It may be noted that the anticomplementary properties of these extracts restricted the concentrations in which they could be used to a

relatively narrow range The method of quantitative complement fixation and the hemolytic system employed were based on the standard methods of the New York State Department of Health [16] The mixtures of antigen antiserum and guinea pig complement were incubated 4 hours in the refrigerator Hemolysis was read against freshly prepared standards and the values for the units of complement required for 50 per cent hemolysis were derived from conversion factors according to the method used by the New York State Department of Health [16]

After a number of experiments for the determination of the optimal conditions it was apparent that as little as 0 05 μg of protein nitrogen of our crude ragweed extract produced measurable fixation of complement With the constant amount of antibody contained in a 1 30 dilution of rabbit antiserum increasing amounts of antigen fixed increasing amounts of complement up to a maximum of seven 50 per cent units with 0 4 μg of antigen protein nitrogen (Figure 1 Curve I)

This sensitive and reproducible method of detection of small amounts of ragweed pollen antigen formed the basis of studies of the human anti

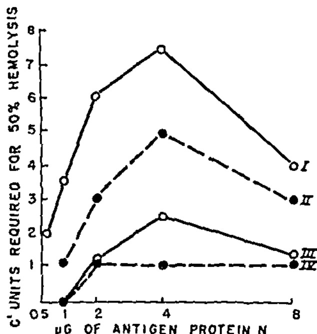


FIGURE 1 The effect of human blocking antibody on the fixation of complement by rabbit antiragweed serum and ragweed pollen extract Curve I rabbit antiragweed serum 1 30 and ragweed pollen extract without human serum Curve II same with human blocking serum (heated 4 hours at 56 C) 1 64 dilution Curve III same human blocking serum diluted 1 16 Curve IV human blocking serum diluted 1 4 From *Journal of Allergy* [9]

ragweed blocking antibody. Human sera to be studied were Seitz filtered and stored at − 0° C. On the day of the test they were heated 4 hours at 56° C. Preliminary studies showed that such sera had no residual complement. The most satisfactory results were obtained by incubating human serum and antigen 1 hour at 37° C, then adding immune rabbit serum and complement and incubating 4 hours at 4° C. After sensitized red cells were added, the tubes were incubated 15 minutes at 37° C and the degrees of hemolysis read.

Under these conditions human sera known by the method of passive transfer to contain blocking antibody produced well marked inhibition of complement fixation (Figure 1, Curve IV). The criterion of inhibition was the occurrence of 50 per cent or greater hemolysis in a mixture containing blocking antiserum with absence of hemolysis in the control mixtures. The results obtained for a serum were expressed in terms of the dilution of serum producing inhibition by this criterion.

The specificity of the reaction was found to depend greatly on the concentration of immune rabbit serum used in the mixtures. The most satisfactory results were obtained with dilutions which fixed six to eight 50 per cent units of complement; in the case of the pools of rabbit sera used, a 1:10 or 1:30 dilution.

The specificity of the inhibition was tested by studying sera of 69 patients who were not allergic to ragweed and had not received injections of ragweed extract. All of these gave negative results, as did the sera of 14 ragweed sensitive patients who had not received injection treatment. The sera of 3 patients allergic to tree or grass pollens and treated with these pollens but not with ragweed also gave negative reactions.

As a further test of specificity, 3 sera which showed definite inhibition of the ragweed complement fixation were tested for their effect on the fixation of complement by an ovalbumin rabbit antiovalbumin system. They had no inhibiting effect on this unrelated system.

The inhibition of complement fixation was compared with the demonstration of blocking antibody by the method of passive transfer,[15] by performing both tests on different portions of 75 sera. These included 20 from persons not allergic to ragweed, 14 from ragweed hay fever patients who had not received treatment, 24 from ragweed sensitive patients in various stages of injection treatment, and 17 from nonallergic volunteers given injections of ragweed pollen antigen for the express purpose of stimulating the formation of blocking antibody. Thirty seven sera gave negative results by both tests. Twenty six blocked 100 to 200 units of ragweed antigen by the passive transfer method; none of these caused inhibition of the complement fixation reaction. Seven sera showed titers of 500 units by the passive transfer method. All of these inhibited the

complement fixation reaction but failed to give definite results when the test was repeated The 5 sera with passive transfer titers of 1000- 000 units showed definite inhibition of complement fixation

It was apparent that the inhibition of complement fixation was closely correlated with the presence of blocking antibody determined by the passive transfer method but that the complement fixation test was less sensitive Sera showing titers of 1000 units or more by passive transfer inhibited the complement fixation reaction while those with titers of 00 or less gave negative results Sera with titers of 500 units were generally positive although of 7 showed questionable reactions The sensitizing antibody in the sera of untreated patients had no effect on the complement fixation reaction

It is believed that the blocking antibody was demonstrated by this *in vitro* technique and manifested its presence by binding the corresponding antigen

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7

Approaches to the Problem of Detecting Antibodies

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INTRODUCTION

Most of the serological tests for antibodies in use today are based on reactions which were first observed in the last century. By 1901 the precipitin reaction, bacterial agglutination and lysis,[40] complement fixation[8] and toxin neutralization[3] had been described. These reactions were first observed not as a result of any deliberate search for antibodies as such, but rather as a consequence of investigations of a general nature on the changes which take place in the blood of animals following infection with various bacteria. It was soon discovered that an infectious agent was not necessary to elicit this kind of response, and that the injection of many foreign proteins into the tissues would be followed by the appearance in the serum of antibodies reacting specifically with the injected protein or antigen. Nevertheless, the study of antibodies and immunology continued for many years to be carried out almost exclusively by bacteriologists.

It is now well known that all the serological procedures mentioned above depend upon some secondary manifestation of the primary union of antibody and antigen; it is also well known that the primary interaction of antibody and antigen is not necessarily followed by any such reaction. Whether or not a secondary reaction is observed depends partly on the nature of the antigen. Obviously, agglutination can occur only when an antigen is on the surface of a particle which can be agglutinated, and lysis can follow only when the antigen is on the surface of a cell which can be lysed. Very small haptenic molecules will combine but not precipitate with their antibodies, although these same antibodies *precipitate proteins to which the hapten is attached chemically.*[9] However, the nature of the antibody is apparently also important in deciding the outcome of its combination with antigen. It has been shown recently that there can exist in human sera three kinds of antibody to diphtheria toxin, all of which neutralize the toxin but which differ from each other in their precipitating, skin sensitizing, and complement fixing abilities and in their physicochemical properties.[55, 57, 70] There is now ample evi

dence for the heterogeneity of antibodies[10, 2, 3, 4, 47, 48] heterogeneity that is not only in the physicochemical sense but also in respect of the consequences of their union with antigen *in vitro*

Thus although the classical immunological procedures are clearly adequate for many purposes particularly in the study of infectious diseases they are certainly inadequate when it comes to measurement of primary antibody antigen interaction This inadequacy has been felt less by bacteriologists than by those concerned with the various diseases and abnormalities which appear to be the consequence of the action of antibodies *in vivo* since in many such cases the important antibodies have eluded detection by the classical procedures In fact one of the biggest challenges in immunology today is the failure up to now to demonstrate *in vitro* the specific host factors or antibodies which are responsible for various types of hypersensitivity

There has thus begun in immunological investigation an era of deliberate attempts to work out methods for detecting antibodies which are not detectable by the classical procedures It is the purpose of this paper to discuss the approaches to this problem However, the subject is so vast that it is necessary at the outset to limit the discussion rather severely in certain respects In the first place only *in vitro* procedures will be described and then mainly those dealing with the detection of interaction between serum antibodies and soluble antigens it will be necessary on the whole to omit reference to methods dealing with the demonstration of cellular or fixed antibodies The emphasis will be on the detection of antibodies rather than on their quantitation and on methods which use only rather small amounts of serum (Since nonprecipitating antibodies are often coprecipitated with precipitating antibody and antigen[41] the quantitative precipitating test[41] can detect nonprecipitating antibodies, but rather large amounts of serum are necessary)

Even with these limitations it will be impossible to mention more than a few of the many various attempts to develop new procedures for detecting antibodies These few examples will be selected as representative of the different types of approach to the problem

Before going further however it may be worth while to try to make a list of the qualities we might wish for in a sort of utopian serological test for antibodies (1) The test should detect specific antibodies in very small amounts (2) It should measure the primary interaction of antibody and antigen and it should not depend upon any secondary reaction which only takes place in certain cases (3) It should be capable of providing quantitative data concerning the amount of antibody present and also concerning its avidity (4) It should tell something about the biological properties of the antibody which it detects being able for example to distinguish between precipitating nonprecipitating and skin sensitizing

antibodies etc (5) It should be applicable to any antibody antigen system (6) It should be simple to perform

We may now pass on to discuss some of the newer methods for antibody detection and see how far any of them goes towards fulfilling the requirements of this ideal test

METHODS DEPENDING UPON SOME SPECIAL QUALITY OF THE ANTIGEN

Many serological procedures for measuring antibodies are by their very nature limited in their usefulness to certain special antibody antigen systems Methods based on the neutralization by antibody of a special biological activity of the antigen come into this category For example specific inhibition of the *in vitro* activity of various streptococcal products (e g streptokinase streptococcal hyaluronidase and streptococcal desoxyribonuclease) has turned out to be a useful aid in the study of the immune response to streptococcal infection in man[63] Interference by antibodies with the biological activities of enzymes[1] and with the infectivity of viruses for cells are other examples of the potential usefulness of such procedures in certain systems

It seems likely that some of these neutralization methods may provide a direct measure of the primary interaction of antibody and antigen at least in cases where a mere combination of the antibody molecule with the biologically active groups of the antigen suffices to prevent the activity Unfortunately the vast majority of important antigens against which we wish to detect antibodies do not have any special known *in vitro* biological activity that can be neutralized so the principle obviously cannot be applied to them On the other hand these procedures do have a usefulness in immunology beyond the obvious one connected with the particular antigen itself Their unusual sensitivity and the fact that they may detect antibodies of types not detectable by classical immunological techniques make them potentially very useful methods for fundamental studies on the nature of antibodies and the mechanism of the antibody response For instance the diphtheria toxin antitoxin system provides a useful model for the study of different kinds of antibody since toxin neutralization appears to be a direct measure for the primary interaction of antibody and antigen[6 57 70] Present evidence suggests strongly that toxin neutralization even detects the antibodies or reagins of the type responsible for the Prausnitz Kustner reaction These antibodies do not fix complement with antigen and do not precipitate (although they coprecipitate)

Perhaps the most sensitive of all *in vitro* serological procedures for the detection of antibody are those based on the ability of antibodies to interfere with the activity of bacteriophages either by neutralizing their

infectivity or as in the case of T_4 bacteriophage by rendering permanent the reversible activation of the phage by tryptophane [46]

One of the most convincing demonstrations *in vitro* of an antibody almost certainly of etiological significance in a hypersensitive state is provided by the experiments of Ackroyd on sedormid purpura a condition which occasionally follows the administration of the drug sedormid (allyl isopropyl acetyl carbamide) In the first place Ackroyd showed that the addition of sedormid to whole blood of patients recovering from sedormid purpura caused lysis of the platelets and that no platelet lysis occurred when sedormid was added to the blood of normal persons This lysis of platelets appeared to be due to a plasma factor since platelets from normal persons lysed when mixed with sedormid and plasma from sedormid purpura patients In fact platelets from normal persons and platelets from sensitive persons were identical in respect to this reaction The plasma factor was not destroyed by heating to 56° C for 30 minutes although complement was necessary for the lysis to occur In the absence of complement platelet agglutination was seen From subsequent studies on the mechanism of this reaction Ackroyd concluded the following points If a mixture of platelets sedormid and lytic factor was centrifuged the sedormid and the lytic factor were both removed with the platelets However neither the lytic factor alone nor sedormid alone formed a stable compound with platelets An important observation was the fact that sedormid could be removed from combination with the platelets and the lytic factor by dialysis in saline As this happened the lytic factor became separated from the platelets Ackroyd concluded from this last finding that sedormid acts as a link between the platelets and the lytic factor

In discussing the mechanism of this very interesting serological reaction and the mechanism of the hypersensitivity Ackroyd postulates that sedormid acts as a hapten conferring antigenic properties on the platelets in respect of their ability to react with antibody *in vitro* He points out that his results would seem to indicate that the compound formed by the union of sedormid with the platelets must be very labile and that it is therefore difficult to see how the sedormid platelets complex can remain in contact with the antibody forming tissues long enough for it to stimulate antibody formation Perhaps he suggests this is the very reason why so few persons receiving the drug become sensitive to it

A rather similar reaction has been observed in mixtures of certain antigens of the tubercle bacillus with their corresponding antisera and normal sheep red cells When normal red cells are added to mixtures of suitable tuberculous antigen preparations and rabbit antisera and complement hemolysis occurs If complement is not present hemagglutination may occur but it is often very weak In the case of guinea pig antisera

hemolysis occurs in the presence of complement while in its absence neither hemolysis nor hemagglutination occurs This fact suggests that the antibody responsible for the serological reaction may be of very low avidity The reaction with tuberculin protein seems to be associated with an affinity of the antigen for the cell surface since all the reactive antigen can easily be absorbed out of a solution by a few successive absorptions with normal red cells However the complex between the antigen and red cell is not a very stable one since the antigen can easily be removed from the red cells by washing them a few times in saline

It is possible that the lysis of leukocytes in the presence of tuberculin and plasma from immunized animals and tuberculous persons and also in certain other antibody antigen mixtures is related to these reactions[34, 2]

It seems very probable that the lytic factor or antibody demonstrated by Ackroyd plays an important role in the syndrome of sedormid purpura From the general immunological standpoint his findings are important *in that they show that an antibody can exist which can have* profound physiological or rather pathological effects but which forms only very unstable complexes with the antigen Thus antibodies may be of very low avidity but nevertheless of great importance in pathogenesis of allergic conditions and any method aimed at their detection must take this into account

The Use of Red Cells in Serology

It would be hard to overestimate the part played by the red cell in serology during the past half century Aside from the important immunology of the red cell itself especially in relation to blood groups one cannot help being impressed by the unique contribution this cell has made to the study of other immunological systems For many years it played a vital role in complement fixation tests for detecting antibodies and in the diagnosis of disease But the observation in 1941 of McClelland and Hare[4] and of Hirst[4] that certain viruses cause hemagglutination which can be inhibited by antiviral serum opened up a new era of service of the red cell in serology There is no need to describe here the advances made in the field of virology which followed the use of red cell hemagglutination and hemagglutination inhibition techniques

It was then found[50, 53, 55] that a few bacteria or their products would also agglutinate red cells and that this hemagglutination could be inhibited by antibacterial sera But of greater significance from the practical standpoint was the observation that many microorganisms produced substances which could be adsorbed onto red cells without causing their agglutination but that the addition of antiserum against the homologous organism

would agglutinate the cells[38] [9] This last observation has proved to be of considerable value in the serological study of many bacteria protozoa and rickettsiae[12] [68] For various reasons these coated red cell techniques have proved much more successful than earlier coated particle methods in which the antigen was adsorbed onto collodion or dye particles[13] [19] [47] or onto bacteria [4] etc

Simple Coated Red Cell Tests

In the simple coated red cell techniques the cells are usually treated with bacterial antigen preparations at 37° C for an hour or so and are then washed The cells are resuspended and added to dilutions of the test serum Hemagglutination occurs when the serum contains antibodies against components of the antigen preparation which had been adsorbed onto the red cell surfaces In the presence of complement hemolysis usually occurs instead of hemagglutination The main advantages of these red cell techniques are their sensitivity and their simplicity According to Grabar[3] such a passive hemagglutination technique can detect as little as 0 003 μg of antibody nitrogen against the adsorbed antigen (in this case the O antigen of *Salmonella typhi*) The hemolytic technique with complement is usually more sensitive than hemagglutination[13]

This type of hemagglutination technique is obviously limited in its usefulness to antibody antigen systems in which the antigen has an affinity for the normal red cell surface Certainly most antigens do not have this property of rendering normal erythrocytes sensitive to agglutination by the corresponding antisera However almost all the bacteria which have been studied in this respect have been found to produce substances usually thought to be polysaccharide in nature which are capable of sensitizing red cells in this way

The passive hemagglutination procedures as described above are capable of detecting only antibodies with agglutinating or hemolytic properties However the Coombs antiglobulin test [6] has been successfully applied in some instances thus theoretically rendering the method capable of detecting nonagglutinating or incomplete antibodies[3]

Coated Red Cell Methods Involving Modification of the Red Cell Surface

Mere treatment of normal red cells with protein solutions does not normally render them sensitive to hemagglutination by the antiprotein sera There are however ways of modifying the red cell surface so that it can be sensitized with protein antigens[11] [49] Pressman Campbell and Pauling attached ovalbumin to red cells by treating the cells first with bisdiazotized benzidine and then exposing them to a solution of the antigen The ovalbumin became fixed to the cell surface apparently being coupled through the second unbound diazonium group From a practical

point of view this method suffered from the disadvantage that the treated red cells were very fragile and lysed an hour or two after sensitization and that the titers of the antisera were not very high

This method has been investigated and somewhat modified by Stavitsky and Arquilla[81] who found it very sensitive They showed for example that red cells artificially coated with insulin in this way were hemolysed by antibody to insulin in the presence of complement[80] The nonspecific hemolysis which occurs in cells treated with bisdiazotized benzidine did not occur immediately and so did not under the conditions of their experiments interfere with the specific hemolysis which they were measuring

Very recently the procedure (as modified by Stavitsky and Arquilla) has been used for the detection of antibodies in human sera against ragweed pollen proteins[74] Four treated patients and 1 untreated patients who had suffered from ragweed pollen hay fever were positive while sera of 13 normal persons gave no reaction The authors suggest that previous failure to demonstrate agglutinating antibodies in the sera of patients suffering from these conditions was merely due to the insufficient sensitivity of the methods used Unfortunately as is often the case in publications on this subject the authors do not state whether the patients had been skin tested with pollen protein True the amount of antigen gaining access to the tissues in a skin test is likely to be rather small but the antibody content of the sera is also very small and as the authors point out it would not be detected at all if the test were not exceptionally sensitive It is not impossible that the small amount of protein gaining access to the tissue in the skin test would be sufficient to cause the appearance of antibodies in persons already sensitive to the antigen while causing no detectable response in normal persons It is known that a skin test with tuberculin will sometimes cause the appearance of antibodies in the blood of tuberculous or BCG vaccinated individuals where there had been no demonstrable antibodies prior to the test[6,17,76]

Treatment of red cells with very dilute solutions of tannic acid changes their surface properties so that the cells adsorb proteins from solution Tanned red cells which have adsorbed a protein in this way are susceptible to agglutination by the antiprotein serum This fact forms the basis of a method for detecting antibodies to protein antigens[22] Most protein antigens can be adsorbed onto tanned red cells and the method is one of the more sensitive *in vitro* serological techniques

A number of investigators have studied the quantitative aspects of the tannic acid test in various antibody antigen systems[9,23,80,81,73,78,79] and it seems that the method is capable of providing quantitative data of sufficient accuracy to be useful in estimating for example small amounts of diphtheria toxin and antitoxin[36,61,73,78,9] The sensitivity of this

system for the detection of antitoxin is of about the same order as the intradermal neutralization test In the case of the antigen ovalbumin it was calculated that the test is capable of detecting 0 005 μg of antibody nitrogen [9]

A recent modification of the procedure [6 09] involves exposing the red cells to formalin before treating them with tannic acid and the antigen The formalin treatment fixes the cells so that after tanning and coatin with antigen they can be stored for many months in the frozen or freeze dried state without loss of sensitivity to agglutination This modification should prove very useful since two important disadvantages of the tannic acid test are the trouble involved in tanning and sensitizing the cells each day and the fact that the sensitivity of the test may vary somewhat from day to day apparently because of some undetermined variation in the state of the red cells

As mentioned above the tannic acid procedure is very sensitive and frequently it shows the presence of antibodies in sera which are negative when tested in other serological tests This fact led to the suspicion that the tannic acid hemagglutination test may detect antibodies of a non precipitating or incomplete type [9 7] Subsequent work however tends to indicate that the method probably only detects 'complete antibody [23 1] One of the disadvantages of the tannic acid hemagglutination test is that the Coombs antiglobulin test for the detection of non agglutinating antibodies cannot be successfully applied to it [23 81]

The Red Cell Linked Antigen Test

Coombs and his colleagues have devised another and very ingenious method for fixing soluble proteins onto red cells [3] They call it the red cell linked antigen test and have shown that it can be used in conjunction with the antiglobulin or developing procedure so that it can detect both agglutinating and nonagglutinating antibodies

In the most recent form of the method [1] the antigen is first coupled by diazotization to nonagglutinating rabbit antibodies against sheep red cells (Forssman antibodies) These nonagglutinating rabbit antibodies are obtained by photo oxidization of an ordinary Forssman rabbit serum [84 1] A solution of the conjugate is then added to the red cell suspension The Forssman antibodies although conjugated with the antigen retain their serological activity and so are specifically adsorbed onto the red cell which thus become simultaneously coated with the antigen Since the antibodies are not agglutinating the coated red cells remain in suspension After washing the red cells are added to dilutions of the test serum (e g human) if the serum contains complete antibodies against the protein antigen hemagglutination occurs The presence of incomplete antibodies in a serum which is negative in the direct test can at least in

some cases be revealed by washing the cells and exposing them to an antiglobulin serum (e g anti human globulin)

This method was first used with egg albumin as antigen Certain human sera were found to agglutinate the treated cells directly while others contained incomplete antibodies detectable only by application of the antiglobulin procedure The agglutinating antibodies but not the incomplete antibodies could also be detected in the tannic acid hemagglutination test Antibodies against egg albumin either complete or incomplete were found not only in sera of persons hypersensitive to egg albumin but also in the sera of about 50 per cent of normal persons [2]

More promising results were obtained with horse dander protein as antigen Antibodies against horse dander were found in 6 out of 6 sera from sensitive persons but not in 15 out of 15 sera of normal persons [24] It has yet to be shown whether the antibodies detected in the sensitive persons were those actually responsible for the hypersensitive state

The coated red cell tests then have many useful characteristics and some of them have become more or less established as methods for detecting antibodies against certain antigens They are mostly fairly simple to perform and easy to read They are often very sensitive and have in many cases revealed antibodies not detectable by other techniques They have been useful not only for the detection of antibodies but also in the antigenic analysis of bacterial extracts etc However they are quantitative in the relative sense only and cannot supply information about the serological reactions which they detect in terms of milligrams of antibody nitrogen and so they are of little use in investigations which require this kind of information

So far it must be admitted there is no completely convincing evidence that these hemagglutination tests can detect any antibodies of the Prausnitz Kustner type or any of the specific factors responsible for delayed type sensitivity

Serological Procedures Based on the Use of Labeled Antibody or Antigen

During recent years there has been a considerable advance in the development of techniques of labeling proteins with radioactive isotopes or with fluorescent molecules and this fact is already proving very useful to immunologists It seems probable that before long as isotopes and apparatus for their measurement become universally available procedures based on the use of tagged antigen or tagged antibody may form the basis of many new routine laboratory procedures for detecting antibodies

The method of Coons [7, 9] involving the use of antibodies labeled with fluorescein isocyanate is well known and needs no description here So far this procedure has been used mainly for locating antibody in the tissues and for the serological identification of organisms and has not been applied to any extent to the problem of detecting antibodies responsible for different kinds of hypersensitivity However it seems very likely that it will provide some information on these problems before long

None of the new methods for detecting antibodies based on the use of tagged antigens or antibodies can be said to be beyond experimental stages although some of them show some promise of general usefulness In principle methods involving the use of radioisotope labels should be capable of detecting extremely small amounts of antibody and also of providing quantitative data about the antibody antigen reactions which they measure

The first big question which had to be considered when labeling techniques were first applied in immunology was whether the labeling procedures altered in any way the immunological activity of the antibody or antigen It is now clear from many studies that antibody can be satisfactorily labeled with for example I^{131} or with fluorescent molecules without impairing its ability to combine with antigen It is also clear that antigens such as human albumin can be satisfactorily labeled with I^{131} without any apparent change either in their antigenicity or in their capacity to combine with antibodies The experiments for instance of Eisen and Keston [31] show that I^{131} labeled BSA can be precipitated by a precipitating antiserum to this antigen This work and also the subsequent investigations of the method by Talmage and Maurer [53] demonstrated the potential sensitivity of labeled antigen techniques for the detection of antibodies

Serological techniques with labeled antigen or antibody aimed at measuring the combining capacity of antisera, are all based on the reasonable assumption that antigen will behave in certain respects differently when combined with antibody than it would do in the free state

Some of the physicochemical properties of the antigen antibody complex for example can be expected to be different from those of the free antigen The complex and the antigen might differ in their solubility in various solvents in their sedimentation rates in the high speed centrifuge or in their electrophoretic mobilities etc Some of the new procedures mentioned below are based on such differences the radioactive label on the antigen being used to trace its physicochemical behavior in various circumstances

Of the various biological properties of antigens the most obvious one to be modified by combination with antibodies is the antigen s capacity

to react with further antibodies This is the blocking principle and two methods will be described below in which radioactive isotopes have been used as indicators of such specific interference by antibody in the test serum Since the ability to react with antibody is a property common to all antigens methods based on this principle can be expected to have fairly wide applications

Differences in Solubility Between Antigen and Antigen Antibody Complexes

Bovine albumin when mixed with normal rabbit serum is not precipitated by half saturated ammonium sulfate However if a mixture of bovine albumin and rabbit antibodies against this antigen is treated with half saturated ammonium sulfate the albumin antigen will be carried down in the precipitate combined with antibody From this fact Farr has developed a serological procedure for estimating the primary interaction between bovine serum albumin and its antibody The antigen is radioactive (labeled with I^{131}) and is added to the test serum When the mixture has been allowed to stand for some time ammonium sulfate is added to half saturation and the precipitate which appears is centrifuged down Radioactive antigen when combined with the antibody is found in the precipitate Farr describes the results of this procedure in terms of micrograms of bovine serum albumin bound to antibody per milliliter of antiserum in the zone of infinite antigen excess and this value is called the antigen combining capacity of the serum

The surprising thing about this technique is that it works From our knowledge of the effect of strong solutions of various sorts of antibody antigen complexes[69] we might expect that the seemingly drastic treatment with half saturated ammonium sulfate would dissociate the complex between albumin and its antibody However Farr's experiments[35] suggest that if anything the ammonium sulfate has a locking effect upon the complexes After precipitation of the ammonium sulfate the antibody does not combine with or release I^{131} BSA

Much more work has to be done on this technique before its potentialities can be assessed It is a sensitive procedure and as Farr has shown it will detect antibodies in the zone of considerable antigen excess where no precipitate appears in the absence of ammonium sulfate as measured with labeled antigen according to the method of Talmage and Maurer[43] Obviously the procedure is limited in its usefulness to the study of antibody antigen reactions in which the free antigen is soluble in half saturated ammonium sulfate As mentioned above it is theoretically capable of detecting nonprecipitating antibodies to albumin assuming that the complexes with such antibodies behave similarly in being precipitated but not dissociated by the ammonium sulfate

Differences in the Electrophoretic Mobility of Antigens and of Soluble Antibody Antigen Complexes

It is well known that soluble antibody antigen complexes have distinct electrophoretic mobilities which are often different from those of the free antigen [6, 7] Marrack *et al* [6] made precipitates of albumin with rabbit antialbumin serum and after washing them redissolved them in excess antigen The solutions were examined in free electrophoresis and were found to contain two components apart from the free albumin apparently comprising two different kinds of antibody antigen complex Since antigen was greatly in excess it could be assumed that all antibody sites were occupied The authors suggest therefore that the two different kinds of complexes were due to the existence in the serum of both divalent and univalent antibodies

The experiments of Berson and his colleagues [4, 5] using I^{131} insulin have shown an interesting application of this principle to the problem of detecting antibodies against insulin If insulin in low concentration is placed on filter paper of the type used for zone electrophoresis it becomes rather firmly adsorbed to the paper at the point of application This adsorption also occurs when the insulin is mixed with normal serum so that in paper electrophoresis of such mixtures the radioactivity remains at the starting point while the serum proteins migrate towards the anode in the usual manner If however this experiment is carried out with a mixture of insulin and serum from a person who has received injections of the hormone then the I^{131} insulin is not adsorbed by the paper instead it migrates along the paper just ahead of the gamma globulin fractions of the serum This is apparently due to the presence of specific antibodies against insulin in the serum since all sera from persons who had received insulin injections three months or more previously behaved in this way while sera from normal persons did not

In starch block electrophoresis the picture is somewhat different since insulin is not adsorbed by starch as it is by paper In normal human serum insulin migrates with a mobility almost as great as that of serum albumin In contrast when mixed with the serum of an insulin treated subject it migrates again with the globulins

Other antibody antigen systems have been examined in this procedure For example if labeled I^{131} human albumin is mixed with anti human albumin serum in different proportions and the mixtures after incubation are submitted to zone electrophoresis on paper the distribution of the radioactivity on the paper depends on the relative concentration of antigen to the antiserum In the region of marked antibody excess radioactivity is found to migrate with the gamma globulins In the case of mixtures made in the equivalence zone all the antigen remains precipitated at the starting

point Soluble complexes again occur in the region of antigen excess migrating with the globulin Free albumin also appears in the region of antigen excess migrating with the albumins of the antiserum Finally in the highest dilutions of antiserum all the albumin migrates as free antigen in this region[6, 77] The total antigen combining capacity as measured by this method exceeds the maximum precipitated antigen by three to six fold[6]

Zone electrophoresis of a mixture of test serum with radioactive labeled antigen can then be used for the detection of antibody It is capable of demonstrating reactions between soluble antigens and antibodies in which no precipitation takes place We may well hope that this approach will turn out to be useful in demonstrating the existence of antibodies of etiological importance in drug hypersensitivities delayed type allergies etc but at present this is no more than a hope Of course the method can be applied only to antigens which have an electrophoretic mobility quite distinct from that of the globulin

The Use of Labeled Antigen or Antibody to Demonstrate the 'Blocking' by Antibody of the Antibody Combining Capacity of Antigen

Two methods will be mentioned under this heading The first is applicable to the study of cellular antigens and involves the use of tagged antibody against the antigen in question The method was developed by Talmage and Freter[8] and was used in the study of antibodies formed in rabbit sera against sheep cells The combining capacity of antisera as measured by this new technique was compared with their hemolytic activity

The method consists essentially of the addition of sheep red cells to mixtures of different dilutions of the test serum with a fixed amount of I[131] labeled antibody against the sheep cells After incubation for a given time the mixtures are centrifuged and the radioactivity of the cells measured When no antibody is present in the serum all the antigen sites on the cells are left free for the labeled antibody most of which is adsorbed onto the cells under the conditions of the experiment However if antibodies are present in the test serum they compete with the labeled antibody for the antigen sites on the cells and so reduce the uptake of labeled antibodies The method is a sensitive one and provides a direct measure of the capacity of an antiserum to compete with another known serum for antigen sites It does not depend on the ability of the test antibodies to cause a secondary reaction of any sort

In its present form this method is applicable only to insoluble antigens (antigens forming part of a cell surface) but it is not impossible that it could be adapted for soluble antigens by rendering them insoluble by some means (e g by fixing to some kind of particle) As Talmage and

Freter[8] pointed out the method is more sensitive for the detection of avid antibodies than nonavid antibodies. This might be a disadvantage in attempts to detect antibodies responsible for hypersensitivities since there is some evidence suggesting that the important antibodies may form only very loose complexes with antigen.

Another new method which has recently been developed is aimed at the detection of small amounts of precipitating and of nonprecipitating antibody against soluble antigens.[15] It involves the use of labeled antigen rather than labeled antibody and depends on the principle that a specific antibody, whether precipitating or not, can combine with the antigen so as to interfere with the capacity of the latter to react subsequently with further antibody. The test is best carried out in test tubes, but for some purposes a simple modification which is carried out on strips of filter paper is satisfactory. Only the filter paper procedure will be described here.

It is necessary to have available a precipitating antiserum against the antigen in question. The first step in the procedure consists of the preparation of a specific antibody-antigen precipitate using this antiserum and the homologous antigen (unlabeled). An appropriate mixture of antiserum and antigen is made in the region of antibody excess and is incubated for a time at 37° C and then left in the cold overnight. It is then washed in saline and finally once in distilled water. Small and equal amounts of the precipitate are placed in a drop of distilled water onto the filter paper strips at point B (see Figure 1) and spread across the whole width of the paper. The paper strips are then allowed to dry and are stored away until required. The amount of precipitate placed on the paper depends on the amount of labeled antigen which is to be used in the test. In the experi-

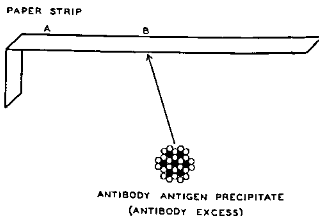


FIGURE 1

ment mentioned below 1 0 μg of precipitate was placed onto each paper at point B 10 μg of this being the albumin antigen

The actual test is carried out as follows A small amount of test serum (e g 100 μl) or test serum dilution is mixed with radioactive antigen (e g 1 μg or less) The mixture is incubated at 37 C for one hour Meanwhile the paper strips (one for each test mixture) are placed horizontally in a plastic box with the bent end dipping into an empty trough After incubation of the test mixtures 10 μl of each is put on the paper at point A (see Figure 1) Immediately this is done the trough is filled with buffered saline which thus wets one end of the paper A cover is then placed on the plastic box The buffer then commences to creep up and along the paper as a result of capillary action When it reaches point A the soluble components of the test mixture are caught up by the moving stream and so migrate along the paper in the solvent front If however the test serum contains precipitating antibodies so that the radioactive antigen in the mixture is precipitated then it will not travel along the paper with the soluble substances of the mixture but will remain at point A If there is no antibody in the test serum then the free antigen will travel in the solvent front until it reaches point B Since the antibody antigen precipitate at point B was made in the region of antibody excess it is bristling with free antibody combining groups ready to absorb more antigen Thus when the free antigen reaches point B it is all absorbed by the precipitate which being insoluble does not move along with the stream of fluid The solvent front thus continues along the paper towards the end having left the radioactive antigen behind at point B

We may now consider a further possibility namely that the test serum contains nonprecipitating antibodies against the antigen In this case the mixture placed on point B will contain soluble complexes of antibody and antigen When the buffered saline reaches point A the moving stream will carry these complexes with it so that no radioactivity remains at point A Since the antigen is already combined with antibody it is not attracted to the unsatisfied antibody valencies of the specific absorbent and so continues on its way in solution in the solvent front

Figure shows some typical results obtained with this technique The antigen in this case was human albumin A precipitating and a non precipitating *rabbit anti human albumin serum* were tested in different dilutions The nonprecipitating serum had been prepared artificially from the precipitating serum by photo oxidization [3 8] In the higher concentrations of the precipitating serum all the antigen remained at point A but as the serum was diluted out so that the antigen was in excess free antigen passed along to point B where it was absorbed by the specific absorbent In the case of the higher concentrations of nonprecipitating antiserum all the radioactivity left point A and was carried past the

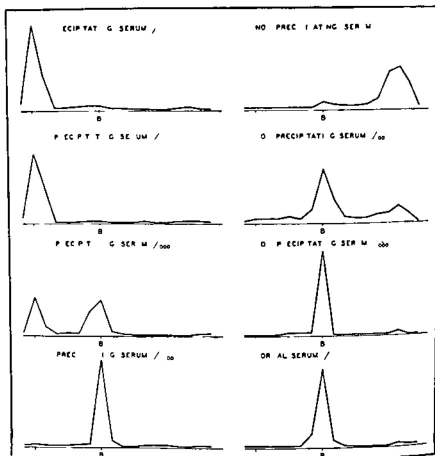


FIGURE Rabbit anti human albumin sera

specific absorbent at point B to the end of the paper indicating that although no precipitate was formed all the antigen was combined with antibody

The general applicability of this procedure has yet to be determined So far it has only been tried with two antigens namely human albumin and diphtheria toxoid and quantitative aspects of the procedure have not yet been worked out in detail As described here it can only be applied to antigens against which precipitating antibodies can be obtained However it is also possible to use instead of antibody antigen precipitate particles coated first with antigen and then with an excess of antibody

The procedure also works satisfactorily from a purely qualitative standpoint if it is labeled with fluorescein isocyanate or with the fluorescent molecule Lissamine Rhodamine B oo (RB oo) [20]

The method is obviously unlikely to detect soluble antibody antigen complexes in the region of antigen excess since the uncombined antigen

groups would react with the specific absorbent in the same way as free antigen For mere detection of antibodies this is not very important since it is possible to work with such extremely small amounts of antigen per volume of antiserum A modification of the procedure involving the use of a specific absorbent consisting of a globulin antiglobulin precipitate in antibody excess (the globulin being from the same species as the test serum) can theoretically overcome this difficulty In this system the antigen is not absorbed by the precipitate unless it is combined with antibody

Comment

There is not a great deal one can conclude from this collection of serological maneuvers While it can be claimed that some of them are especially sensitive and some seem able to detect antibodies not detectable by classical procedures they have not on the whole taught us a great deal yet about mechanisms of hypersensitivity and none of them gets very far towards providing us with our hypothetical utopian serological test

Although the methods involving the use of red cells have certain practical applications it seems that ultimately procedures based on the use of labeled antigen hold best hope for measuring even quantitatively the primary interaction of antibody and antigen in many systems and they are potentially very sensitive

However in many cases there must be further advance in methods of purification of antigens before these techniques can be useful It seems unlikely for example that much progress will be made towards the *in vitro* analysis of serum factors responsible for hay fever before the different proteins of the pollen extracts can be separated so that antibodies can be measured separately against each

Many of these methods described above are aimed at detecting the combining capacities of test sera for antigen Although we know that there are important differences between circulating antibodies of the classical type and those which we are trying to detect responsible for hypersensitivity we nevertheless tend to take the classical antibody antigen reactions as a model for our thinking of interaction between antibody and antigen in hypersensitivity Even in the case of hypersensitivity of the tuberculin type where there is no shred of convincing evidence for anything like a globulin antibody being responsible for the reaction we still tend to be looking for something in the tissues which will combine with antigen much as circulating antibodies can be shown to combine with tuberculoprotein It is certainly difficult to imagine any kind of reaction as specific as the tuberculin test not being associated

with the presence of some host factors with a specific affinity for antigen But the interaction between these host factors and the antigen may be very different from the classical antibody antigen reactions It might involve for example only a very transient relationship which our methods of measuring combining capacity would miss completely

A still more pessimistic view from the standpoint of those of us who are trying to detect antibodies responsible for delayed or tuberculin type sensitivity is that the sensitivity may be due not to the presence but rather to the absence of certain factors We could suppose that many bacterial proteins are potentially toxic for cells but that under normal conditions the cells contain naturally occurring protective factors having some degree of specificity * Infection with certain bacteria might result for one reason or another in the appearance of cells devoid of these more or less specific protective factors The degree of sensitivity to tuberculin could be thought of as a reflection of the number of cells present which are devoid of the factors protective against tuberculo protein Polysaccharides are perhaps not potentially toxic for cells so that delayed type sensitivity to them cannot exist We can however receive some cheer from the fact that although this view cannot be completely discarded at present there are a number of recent experimental reports which at least at first sight tend to speak against it

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DESIGNATED DISCUSSION

DAN HAMILTON CAMPBELL (Pasadena California) I wish to compliment both Dr Boyden and Dr Sehon on excellent stimulating papers Dr Boyden raised many interesting questions—particularly when is an antibody not an antibody However since time is short and I have been more actively engaged in the field that Dr Sehon has presented I should like to reserve my few comments and questions for him

My first question is how much antigen and antibody are actually involved in tests with antigen coated red blood cells and my second question involves the data on the molecular weight of reagins

I as well as many others am particularly interested in the molecular weight or size of reagins since several years ago Dr Wiener proposed that the Rh agglutinating antibodies did not pass through placental barriers whereas incomplete antibodies did because of differences in size We thought this idea to be a bit naive but worth testing so we treated Rh antiserum by high speed centrifugation and found that the pellet centrifuged down contained most of the agglutinating antibody but very little of the incomplete or blocking type Perhaps Wiener was correct so we turned our attention to another type of antibody namely reagin which also supposedly does not pass placental barriers Carrying out the experiments in essentially the same manner in which all the heavy molecular weight antibody was collected as a pellet after several hours at high speed we failed to find any relative concentration of reagin in the heavy fraction from 15 positive pollen sensitive serums I believe the relation of molecular size of protein to placental permeability is an important problem and must be carefully studied before any definite conclusions are made

Turning for a moment to the first question I raised regarding the tests with antigen coated erythrocytes I should like to point out the extreme sensitivity of cells treated with various reagents including bisdiazotized benzidine or tannic acid For example we have found that such sensitized cells will agglutinate with very high dilutions of soluble antigen antibody complexes using either ovalbumin or bovine serum albumin as antigen and rabbit as a source of antibody Perhaps antigen and antibody are playing some role as a specific immune mechanism in these reactions However the possibility of nonspecific factors or even soluble antigen antibody complexes which might be present in serums must also be considered

GENERAL DISCUSSION

DR SEHON I am glad that Dr Dan Campbell brought up these other points

Regarding the sensitivity of the method I should like to refer to the reversed hemagglutination experiment in which we coupled antibodies to red blood cells We found that the antigen could be detected in extremely small quantities The amount of antigen which still led to the aggregation of red cells coated with antibody was of the order of 10 mg of material (which is equal to 10^{-4} μg of the material) This would mean that this method is about 1000 to 10 000 times more sensitive than the agar gel techniques

There is no doubt in my mind that this method is highly specific The specific factors involved can be detected readily and unambiguously by using the proper controls namely a serum from a nonimmunized animal or from a normal individual in the same dilutions as the serum containing antibodies

The method used in our laboratory is similar to Dr Boyden s technique (i e tanned red cell technique) In this connection may I say that Dr Boyden has also found high titers in the order of millions However in his technique the antigen is coupled to the red cells by rather non specific adsorption forces which are in all probability hydrogen bonds provided by the tannic acid In consequence one would expect that in his method some of the antigen under certain circumstances might become desorbed and as a result the sensitivity of the test would be decreased In contrast in the BDB method the antigen is coupled by stable covalent azo bonds which cannot be broken off by any simple treatment Incidentally the dissociation energy of these bonds is probably about 150 kcal /mole I am referring here to the energy of breaking a covalent N=N bond and not to the heat of association for the reaction between antigens and antibodies which has been referred to vaguely in this discussion as the energy of the bond between these entities My criticism of the tanned cell technique finds confirmation in the results of Drs Feinberg and Flick who used Dr Boyden s method for detecting antibodies in allergic sera containing both sensitizing and blocking antibodies They have actually found that ragweed antigens were eluted from the surface of the red cell In consequence partial or total inhibition of clumping will result as demonstrated in our experiments

I don t know where I stand with regard to one of the questions which Dr Campbell raised I am referring to his doubt as to skin sensitizing antibodies being associated with the serum components having a sedimentation constant equal to or higher than 19S All I can say here is that

this is the case in our hands and this is an expression I don t like because it is used so often in so many cases when we refer to biological experiments I should like to have at least *in vitro* experiments conducted in such a way that they can be done by all hands and that all hands obtain the same results As I said before we used the partition cells both with the moving and fixed partition

The reagin we were looking for was reagin to ragweed May I ask Dr Campbell what type of reagin he has used

Dr Campbell Do you mean that the reagin of ragweed pollen is different from reagin to other pollen My point is are you distinguishing reagins now on the basis of source and type of antigen

Dr Sehon Yes I do distinguish them on the basis of source in view of the diversity of antibodies which we have heard about I think this is very important Can you answer my question

Dr Campbell You have big antibodies in Montreal and ours in Pasadena are small

Dr Sehon No we are on the same continent and probably there is the same type of ragweed pollen both in Montreal and Pasadena Are you talking about ragweed pollen or other antigens

Dr Campbell I think our work was mostly with grass reagins

Dr Sehon How many sera have you done

Dr Campbell Approximately fifteen

Dr Sehon There is a question of doubt here and I should like to re establish my credence in our own experiments

Dr Campbell It isn t in doubt I am merely trying to understand the factors in your data and why the two methods should give such discrepancies

Dr Sehon Dr Campbell I learned most of the techniques in your laboratory in 195[illegible] All I can say here is that with the partition cell as shown in one of the slides you can follow visually the migration of the peaks as they move downward to the bottom of the cell As I told you Mr Gyenes and Mr Gordon are filling up the cell with the reaginic serum and they watch the peaks as they move across the barrier As soon as the

peals move across the barrier they stop centrifugation (the barrier moves back to its position of rest in the moving partition cell) One removes first the material from the upper compartment and then both compartments are tested for the presence of skin sensitizing antibodies

In view of the high dilutions in which skin sensitizing antibodies can be detected we should be able to detect them if they are in the upper compartment We don t detect them We detect them only in the bottom compartment That is evidence as far as I am concerned that they are materials associated with serum component traveling with sedimentation constant equal to or higher than 19S

On the other hand if we use antisera to ragweed produced in non allergic individuals — i e in volunteers devoid of skin sensitizing antibody — and if we do the same trick we find that blocking activity and hemagglutinating activity are associated with both the upper and bottom fraction We have not done this sort of experiment only on one serum but on several sera and on one serum we have done it five times Therefore I feel that these two types of antibodies are different also with regard to their sedimentation constants I hope that we shall reach the point where identical results are obtained in one laboratory as in another

Frederick Aladjem (Los Angeles California) I should like to point out that it is perhaps the very sensitivity of the method that may be its pitfall Most people will agree that skin sensitizing antibody may combine with antigen in the test tube But in addition to skin sensitizing antibody most if not all allergic sera contain small amounts of agglutinating and blocking antibody What you are measuring might simply be the small amount of agglutinating antibody present in allergic serums I should like to suggest that you try to separate agglutinating (or precipitating) from skin sensitizing antibody As you know we did this not long ago and found that precipitating and skin sensitizing antibody can be separated

Note Added in Proof (February, 1959) Drs D H Campbell and A H Sehon jointly communicate that on the basis of further experimental data obtained in their respective laboratories the diverging views expressed by them at the meeting in Detroit in March 1958 might be reconciled if the skin sensitizing antibodies were associated with serum components having sedimentation constant(s) intermediate between S7 and S19 Experiments are being designed to characterize more specifically the skin sensitizing antibodies with regard to their sedimentation properties

III

Effects of Antibody and of Antigen-Antibody Complexes on Intact Cells and Whole Organisms

Chairman Walter J Nungester M D (*Ann Arbor Michigan*)

8

*Some Cytologic Effects of Antibodies**

HARRISON LATTA, M.D.
(Los Angeles, California)

The reactions of cells to antisera appear to be special cases of immune processes in the body. These processes encompass a manifold group of reactions that ranges from immunity to hypersensitivity. On one hand there is resistance to the entrance, spread, and growth in the body of viruses, bacteria, cancer cells, and transplanted homologous tissues, while on the other hand there are hypersensitive reactions involving these or simpler agents such as proteins and organic — or even inorganic — chemicals. The terms "immunity" and "hypersensitivity" are usually viewed from the egocentric viewpoint of what is "good" for the animal or human body. It has been difficult, however, to escape the idea that basic mechanisms of antigen-antibody combination in immunity and hypersensitivity are similar. It is becoming increasingly clear that some of the variability in response to antigens depends on diversity of antigens and on diversity of antibodies.[2] Host factors are more obscure but probably at least equally important. Their importance is well demonstrated by those cases in which the same antigen may produce in the same body, and depending on the conditions of its introduction, either an immune or a hypersensitive response.

One hypothesis that attempts to clarify these various responses is concerned with events directly affecting body cells. When a foreign substance first enters the body and some part of it penetrates some of the body cells, it becomes involved in the *metabolism* of these cells. When these or cells subsequently involved form antibodies and release them into the body fluids, the antibodies combine with the available specific antigen. This tends to prevent any deleterious effects of the foreign substance and results in *immunity*. Phenomena of *hypersensitivity* are considered to result if antibodies become bound or adsorbed to the surface of cells and antigen combines with them there, resulting in damage to the cell. It seems to make little difference whether the process is reversed and antigen adsorbed on cell surfaces combines with antibody from the

* Investigations reviewed here have been supported by the Jane Coffin Childs Memorial Fund for Medical Research and the National Institutes of Health, U.S. Public Health Service (Grants C-3304, RC-4262, and H-2194).

surrounding fluid The mechanism just described appears to represent a group of phenomena composing *immediate hypersensitivity* *Delayed hypersensitivity* may be considered to be the result of antigen antibody combination also but with this difference the antibody seems to be intimately bound to body cells and does not appear in the circulation [6] This may be related to the chemical complexity of the antigens All types of cells tested in tissue culture from animals with delayed hypersensitivity react with antigen or hapten This is in contrast to the failure of antigen to inhibit growth in tissue culture of cells from animals with immediate hypersensitivity [3] Increased pinocytosis however has been noted more recently in cells from immediately sensitized animals exposed to antigen[4]

In approaching experimentally the problem of the cellular mechanisms of the body's reactions in different manifestations of hypersensitivity



FIGURE Antiserum hemolysis Sheep erythrocyte 12 minutes after guinea pig complement has been added to cells sensitized with rabbit hemolysin The hemispherical shape of the erythrocyte shown by the shadow from metal evaporation demonstrates that the hemolyzing cell tends to maintain its spherical shape in spite of the forces of surface tension during drying The fine irregular cracks in the surface of this cell were found only in hemolyzing or hemolyzed cells The crystals were precipitated from salts in solution which was held by the spherocyte and the supporting film during drying The rigidity of the spherocytes is in contrast to the elastic surface exhibited by osmotically hemolyzed cells as shown in Figure 2 In Figures 1 and 2 the dark shadows are on the left side of raised structures and on the right side of depressions and holes The metal evaporated at an angle onto the surface has been held in place by a transparent film following which the original cells and film were dissolved a 1y Electronmicrograph ×16 000

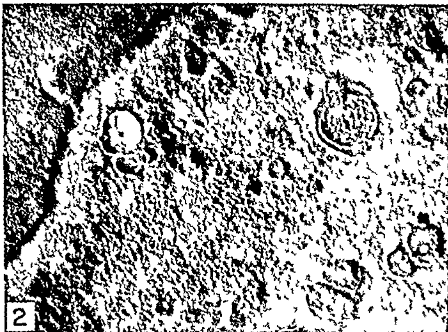


FIGURE 2 Osmotic hemolysis Rabbit erythrocyte lysed in distilled water The red cell ghost lies flat and the surface defects are large and circular in contrast to the cell in Figure 1 Through one hole can be seen a second membrane and another hole probably representing the other side of the cell × 35,000

several questions may be asked of which three have been touched upon in our studies

1 What cell types will react with antibodies? Answering this question in the intact animal is difficult For this reason blood cells and tissue cells isolated in tissue culture have been used

How is the cell damaged? Investigation of the mechanisms of such subcellular events requires a combination of biophysical and biochemical techniques in which phase and electron microscopy are valuable

3 What factors affect the reaction? An answer to this question would not only clarify cell mechanisms but should lead to preventive and therapeutic measures

In delayed hypersensitivity the reacting cells are described as becoming necrotic both in the body and in tissue culture but the cytologic changes in these early experiments are not described in detail [3] A more detailed study for comparison with the following experiments would be interesting

In immediate hypersensitivity mediated by soluble antibody reactions of sensitized isolated cells have been difficult to obtain The closest approach has been with strips of intestinal or uterine muscle Antigen

FIGURE 3 Normal control. Chick-embryo heart fibroblasts after exposure to normal guinea pig serum (1:5) for 3 hours. The cells appear quite normal with the Jacobson and Webb staining procedure. The contrast of the stained and unstained cells in Figures 3 and 4 has been enhanced by photography; the dark interphase chromosomes ... ×650

FIGURE 4 Antiserum. Chick-embryo heart fibroblasts exposed to guinea pig antiserum (1:512) for 3 hours. The nuclei of the damaged cells shrink greatly. Most of the nuclei show one or two vesicular bodies intimately related to the nucleus. The colors of the stain suggest a loss of RNA from the cytoplasm and

antibody complexes have stimulated relatively little immediate reactivity in the body apart from antibody formation.[3] Hence the cytologic evidence rests mainly on the action of antibody and accessory serum factors on cells.

Discussion of Experiments

Erythrocytes

Considerable information has been obtained from classical studies on rates of hemolysis and changes in volume of red blood cells. Simple osmotic hemolysis has been contrasted to hemolysis with a large number of hemolytic agents.[22] With these agents the biconcave red cell becomes a sphere of the same volume, which necessitates a decrease in surface area. Then swelling occurs until a critical volume, different for each agent, is reached, at which point hemolysis occurs. Antiserum seems to belong to



Figure 5. Antiserum — very early reaction. Chick embryo heart fibroblasts after exposure to antiserum (1:56) for 15 minutes. The reaction usually proceeded more rapidly. A few short and swollen rodlike mitochondria are visible, especially near the nuclei. The lower cell shows retraction of its cytoplasm, leaving thin filaments where it was attached to adjacent cells and the glass on which it grew. Note the thin tongue of cytoplasm at the top of the picture. Bright phase contrast. ×1500. (Figures 5 and 6 are reproduced with permission from *Laboratory Investigation*, see reference 14.)



FIGURE 6 Antiserum late reaction The same cells shown in Figure 5 but after hours exposure to antiserum The tongue of cytoplasm at the top has become rounded and swollen The mitochondria have disappeared and only round bodies and vesicles can be seen in the cytoplasm The nuclei and nucleoli have shrunk There is an increased density at the nuclear membranes Nuclear blebs are on the nuclei at the right and the bottom (arrows) × 1500

this group of agents but hemolysis appears so rapidly after the spherical form is reached that a definite volume change has not been measured

In an electron microscope study of the changes during hemolysis with antibody and complement [0] it was found that red cells developed and maintained after drying a rigid surface with numerous irregular defects (Figure 1) Such defects did not appear in the absence of complement Agglutination of red cells increases the appearance of a different type of defect [3] Loss of the elastic surface found normally or with osmotic hemolysis (Figure 2) suggested an increase in intermolecular forces at the surface This supported the classical concept that antibodies act at the surface of cells and suggested the hypothesis of a two step process with the surface changes leading to a spherical shape after which swelling with eventual hemolysis occurs The most apparent swelling force would be osmotic Studies [14] with a hemolytic agent of similar type resorcinol but with a slower action and one that allowed marked volume changes confirmed the concept of increased internal osmotic pressure being the final step in the bursting of the cell The inhibition of resorcinol hemolysis by sucrose in a concentration that balanced the stated internal osmotic

pressure due to hemoglobin confirmed other work [6] indicating that hemolytic swelling could be explained by loss of the normal differential permeability of the red cell membrane to ions Such a loss of permeability would permit ions to reach equilibrium concentrations between the interior and exterior of the cell and allow the osmotic pressure of hemoglobin to exert its swelling force

Tissue Cells

To examine the immediate hypersensitive reaction of tissue cells chick embryo heart fibroblasts grown in tissue culture were used It would have been preferable perhaps to have utilized endothelial or smooth muscle cells because of their role in hypersensitive reactions in the body However several attempts to grow these cells in tissue culture for such experiments have been unsuccessful [11] Fibroblasts represent a general body cell type and appear to be involved in rheumatic lesions especially in mitral valvulitis Moreover injection of labeled foreign proteins into rabbits had demonstrated the remarkable readiness with which localization occurred in mitral valves among other sites and in both macrophages and fibroblasts [1] Because of the failure of previous attempts to elicit a major reaction by adding antigen to cells grown from sensitized animals [3] the reverse experiment was tried with eventual success That is antibodies against cell antigens were produced in guinea pigs and added to the cells in tissue culture Other investigators had used cell antigens to produce antisera against various body cells [4, 7, 9, 1]

The *antiserum* when added to fibroblasts in tissue culture produced an immediate reaction which became apparent in 5 to 15 minutes and developed over 1 5 to 3 hours [13] The antiserum was active at a dilution of 1 51 and required the presence of heat labile complement like factors The antiserum appeared to be species specific but not organ specific It did not affect mouse fibroblasts or human HeLa cells but it did react with chick fibroblasts from spleen and liver and epithelial cells from chick intestine [11] Varying degrees of organ [4] [4] and species [4] specificity have been reported with other antisera The diversity of such antisera is also demonstrated by the lack of correlation between cytotoxic activity and precipitating antibodies The failure of cortisol (hydrocortisone) to inhibit the *in vitro* reaction to antibody and complement [13] is direct evidence indicating that its site of action in the body lies elsewhere probably in vascular or connective tissue [5]

Passive sensitization of tissue culture cells was attempted with complex cell antigens with purified bovine serum albumin or with antisera but with little success [11] Passive sensitization of red cells with adsorbed or chemically bound nonerythrocyte antigens has been demonstrated repeatedly [2]



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The *cellular reaction* finally results in markedly altered dead cells with greatly shrunken nuclei (Figures 3 and 4). The reaction begins sooner and becomes more extensive in flattened cells at the edge of the growth zone which suggests that the surface area exposed to the antiserum is a critical factor. Staining procedures[8] indicate a loss of ribonucleic acid (RNA) from the cytoplasm and a separation of protein from the nuclear desoxyribonucleoprotein (DNP). Similar nucleic acid changes have been described in pyknotic necrosis.[15] The observation of large spherical vesicles lying in contact with many nuclei and even indenting them becomes more meaningful when electron microscopic sections of reacting cells are studied.

The *phase microscope* allows the reaction to be studied while it is occurring in the living system.[13] The earliest changes are retraction of the cytoplasm and swelling of the mitochondria (Figure 5). The retraction is accompanied by swelling of at least part of the cell so that a cytoplasmic bleb or blister is formed. The mitochondria swell and fragment until they become unidentifiable. Some of the lipid droplets seem to increase in size possibly by amalgamation of smaller ones. After the cytoplasm shows considerable change the nucleus begins to shrink attaining finally an irregularly rounded form with a diameter less than half and consequently a volume less than an eighth the original (Figure 6). The thickness of material at the nuclear membrane increases and the nucleoli become smaller and denser.

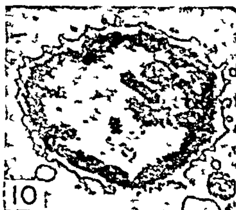
Electron microscopic studies[11] confirm and extend the results found with phase microscopy. Considerable difficulty was experienced in trying to attain high resolution of fine structures in the thin reacting cells. Such difficulty is probably due to the same unknown factors that obscure or destroy detail in the outermost cells of tissue blocks which otherwise are considered at present to be well fixed in deeper layers. The results were improved however by growing the tissue in a plasma clot (Figures 7 and 8). The clot delayed the reaction so that there might be an interval of 45 minutes to one hour before changes in response to the antiserum were apparent. The delay may be attributed to the diffusion time for antibodies to penetrate the clot. The slowness of the subsequent reaction shows that once started it does not automatically proceed at a rapid rate. This is more evidence for diversity of cellular responses to antisera.

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reticulum (er) or α-cytomembranes and lipid droplets.[1] The plasma membrane appears as a thin osmophilic line with fibrillar material closely applied in some areas. In places these fibrils have the periodicity of collagen. The plasma clot has a much finer fibrillar network (Figure 9). *It has been lysed between the* cells here. Cells exposed to fresh normal guinea pig serum have a similar appearance.

These cells at the edge of the growth zone have been fixed in buffered osmium tetroxide, embedded in methacrylate and sectioned in a plane parallel to the glass on which they were grown. Electronmicrograph × 5000.


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The electron micrographs show that the plasma membrane becomes disrupted following the action of antiserum (Figure 9) It cannot be determined whether this physical effect appeared while the cell was reacting or during subsequent fixation and embedding but it was not found in adjacent cells that had not reacted Therefore it can be regarded as a disruption of the membrane consequent to the antiserum action This is analogous to the response of red cells The rodlike mitochondria and endoplasmic reticulum swell and frequently assume a vesicular shape that makes it difficult to distinguish the original type of organelle If internal material or remnants of the cristae are seen within the vesicle a mito chondrial origin is indicated Although the lability of these organelles is well known[1] the changes here are extreme Endoplasmic reticulum seems to produce small vesicles as well as large irregular bizarre forms Lipid droplets show internal irregularity and stain more lightly than those in cells not exposed to antiserum but interpretation is difficult

The *nuclear changes* are dramatic With nuclear shrinkage the granules of the nucleoplasm supposedly containing desoxyribonucleoprotein pile up inside the nuclear envelope (Figure 10) This may correlate with separation of DNA from desoxyribonucleoprotein and with contraction or solvation of the nuclear gel The separation of the outer nuclear mem brane from the inner to form one or more blebs (Figures 11 and 1) is a remarkable finding that explains the perinuclear vesicles seen so fre quently with light microscopy[13] This swelling of the space between the nuclear membranes demonstrates that this perinuclear space has a reac tivity similar to the endoplasmic reticulum and supports the idea[19 1 28] that the endoplasmic reticulum may arise from the nuclear membrane and act as a transport mechanism from nucleus to cytoplasm Nuclear pores seem to disappear during the formation of nuclear blebs (Figures 10 11

FIGURE 8 Normal cell Nuclear pores in tangential section appear as rings about 700 Å in diameter (A few of the pores are indicated by arrows) The nucleo plasm lies in the lower right corner The inner and outer nuclear membranes do not show in the region of a tangential cut Note the relative closeness of the pores in this normal cell in contrast to their apparent sparsity following antiserum (Figures 10 and 11) ×16 000

FIGURE 9 Anti erum The cell on the left shows marked changes when compared with the cell on the right which has been protected from the action of anti serum by cells on each side of it The plasma membrane of the cell on the left is disrupted The cytoplasm is less dense and less granular The fragmented mitochondria (m) now appear as rounded vesicles The endoplasmic reticulum (er) appears as large rounded or irregular vesicles with clear centers The nucleus has not yet reacted The plasma clot appears in the upper left and lower right corners ×4300

FIGURE 10 Antiserum — late reaction Nuclear granules have moved toward the inner nuclear membrane as the nucleus shrank The outer nuclear membrane has separated widely from the inner membrane all around its circumference ×9700

FIGURE 11 Antiserum Nuclear blebs are formed by swelling of the perinuclear space Note similarity to the swollen endoplasmic reticulum ×[illegible],000

FIGURE 12 Toxic normal serum The relation of nuclear blebs to nuclear pores (arrows) is well demonstrated Fixation with potassium permanganate reveals cell membranes with greater clarity than with osmium tetroxide fixation although nucleoproteins and other materials are lost ×8400

and 1) Some pictures show what may be remnants of nuclear pores at the inner nuclear membrane forming the base of a bleb (Figures 11 and 12) If this interpretation is correct it means that as the membranes separate the pores in each are obliterated Although this may seem remarkable there is other evidence that the nuclear envelope is a dynamic structure[1, 1, 2, 25] The reality and relatively large size of nuclear pores are indicated by the penetration of proteins into isolated nuclei[1] Blebs have been observed on nuclei in various salt solutions especially in magnesium chloride or calcium chloride[2] The enhancement of antiserum hemolysis by these ions may indicate a similar mechanism of action on cell membranes

The swelling of cytoplasmic components and portions of the nuclear envelope with a tendency to assume a spherical form suggests *osmotic pressure* as the most likely swelling force Anderson s experiments[1, 2] indicate that the nuclear membrane as a whole is not a semipermeable membrane and that the nucleoplasm reacts as a gel However volume changes in a hydrated gel may be regarded as basically osmotic

Summary

Confirmation of the concept that antiserum unleashes successive osmotic forces within cell components comes from the production of similar changes by hypotonic solutions and distilled water[11] The presence and significance of normally high osmotic pressures in liver and kidney cells have been investigated and discussed[18] The data are consistent with the idea that antiserum acts at the surface of the cell although some internal action cannot be excluded

The cytologic reaction of cells to antiserum seems to be one of several possible patterns The reactions to antiserum to hypotonic solutions and to one control guinea pig serum which was toxic in low dilution were similar This similarity and the resemblance by light microscopy to the pyknotic type of necrosis both suggest that the pattern of cytologic changes cannot be used to diagnose a hypersensitive cellular reaction Differences from the results of others[8] may be due to variability of anti sera in producing injury and in the degree to which particular cells manifest that injury

The reactivity of fibroblasts to antiserum *in vitro* suggests that these cells may react directly in the body with an immediate type of hyper sensitivity or with a disease involving the connective tissue

If osmotic factors play a basic role in hypersensitivity as these experi ments suggest osmotic force may offer a point for general therapeutic attack

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DESIGNATED DISCUSSION

JOHN W REBUCK (Detroit Michigan) Because of the relatively few reports on the subject of Dr Latta s stimulating and fruitful presentation concerning antigen antibody structural relationships and their anatomically disruptive effects we feel it is pertinent to present our related experiments *

Electron microscopy of control human erythrocytes surrounded by their own plasma revealed that desiccation resulting from electron bombardment produced shrinkage of erythrocytes from the plasma (Figure 1) A smooth erythrocytic surface eventuates When human erythrocytes are bathed in plasma containing specific antibodies or panagglutinins and similarly studied the erythrocytes and the proteins again shrink away from each other As a result of this translocation antibody adherent to antigenic structure on the erythrocytic surface is drawn out into a fibril or band These fibrils pull on the antigenic structure of the erythrocyte causing deformation in the form of cones (Figure) and umbilicated plateaus (Figure 4) Although the greater part of the cone is thus of secondary origin its summit is a reacting site flattened at the plane of adherence The simplest reacting sites that we have observed (acquired hemolytic anemia in the adult) possessed summit widths of approximately 300 A [7, 8] Anti Rh antibodies produced somewhat larger but less frequent distortions in the form of umbilicated plateaus [7, 8] Plateau like summits 6 to 4 times the width of the simplest reacting sites were produced by the action of anti A antibody upon Group A erythrocytes [7, 8]

Romanowski and Feltynowski [9, 10] confirmed these preliminary findings for Group B erythrocytes in serum containing anti B agglutinins After desiccation bridges were formed connecting elevations on the agglutinated erythrocytes now separated by shrinkage spaces The widths of the elevations were of the same order as the simplest reacting sites previously described However they felt that the agglutinogens and agglutinins formed a film distributed over the entire erythrocytic surface precluding determinations of antigenic locations Hill and Haberman [1] visualized red cells that had been sensitized by anti D cryptagglutinoid and agglutinated by Coombs serum Where bridges of material were attenuated between the erythrocytes at several points a chain of minute ovoid structures were seen after shadow casting with palladium

Minute elevated segments of the surface ultrastructure (free erythrocytic surface Figure) produced by prior adherence of delicate strands of plasma (autoagglutinins) bridging the plasma-corpuscular shrinkage space afford sharp delineation of receptor sites 210 to 515 A in width In

* With the technical assistance of R M Sturrock



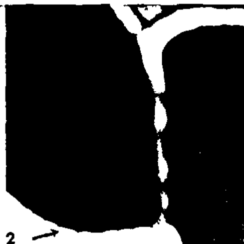






FIGURE 1 A portion of a control erythrocyte Group A_1 with plasma containing anti B agglutinins Osmic acid vapor fixation Note the smooth corpuscular surface and the clear plasma-corpuscular shrinkage space × 14 000

FIGURE 2 Agglutinated Group A Rh (D) positive erythrocytes from a patient whose plasma contained panagglutinins (acquired hemolytic anemia) Osmic acid vapor fixation See text for discussion of the minute segmental elevations of the free surface ultrastructure × 9000

FIGURE 3 A portion of a test erythrocyte Group O Rh_1(D) positive exposed to maternal anti Rh antibody and after washing to rabbit anti-human globulin antibody Osmic acid vapor fixation Arrow points to two receptor sites × 14,000

FIGURE 4 A portion of a newborn erythroblastotic male infant's erythrocyte Group O Rh_0(D) positive in its own plasma containing maternal antibodies against the infant's cells Note the pulled-out surface segments Osmic acid vapor fixation × 14 000

comparison, less specific attenuations between the cells (intererythrocytic material Figure -) are inconclusive in determination of antigen location We suggest that trapping of nonspecific plasma at the erythrocyte plasma erythrocyte interfaces, which may be of aid in agglutination itself, tends to mask the clear cut delineation of receptor sites seen when only the erythrocyte plasma interface is studied Visualization of erythrocytic ghosts agglutinated by their specific antibodies (Rh_0 anti Rh_0 D anti D[7, 8] AB anti-A and anti B[9, 10]) has been substantiated by the formation of hemolysins produced by the injection of well washed human erythrocytic ghosts into rabbits[6] Zacek and Rosenberg[12] perforated the erythrocytic surface with 24 000 r and depicted a fine fibrillar structure to which a few small spherical structures were still attached in the perforated area Aggregates of these same globular formations were so dense on the unaffected membrane wall that they not only gathered on the underlying protein fibrils but filled the spaces between the individual fibrils By a *combination of chemical and serological methods* McKerns and Denstedt[4, 5] were able to demonstrate definitive antigenic localization on the red cell surface Prior occupation of all A receptor sites with anti A did not prevent adherence of anti M to M sites still open on the same erythrocytes Hillier and Hoffman and their fellow workers[3] have afforded the most exact ultrastructural basis for pursuit of antigenic plasma membranes Such membranes are covered with plaques roughly cylindrical approximately 30 A thick, with a diameter varying between 100 and 500 A Lipids are involved in their attachment to the underlying fibrous component of Wolpers[11] and are presumed to line the potential pores between the plaques

Confirmation of a specific topographic localization of erythrocytic antigens is furnished by consideration of erythrocytic behavior in a positive Coombs test Figure 3 depicts a portion of a test erythrocyte (Group O Rh_0 D positive) exposed first to maternal anti Rh antibody which had proved lethal for her fetus and secondarily after washing to rabbit anti human globulin antibody Bands of double antibody containing plasma are adherent to infrequent intermediate sized plateaus Closer examination of the summit surface (Figure 3 arrow) reveals that the actual receptor sites are at either end of the summit and measure 260 and 350 A in width respectively They are separated by a nonreacting surface in between which has not bound plasma It is apparent that an umbilicated or scalloped plateau will be produced when two or more antigenic sites are closely grouped The actual receptor sites on the flanking plateaus are masked by complete adherence of the plasma bands This double banding of the antibody in the Coombs test greatly facilitates receptor site detection because of the infrequent breakage of the stout bands across the artefactual shrinkage space

Further support of our interpretation was furnished when direct electron microscopy was performed on the erythrocytes of a newborn male infant* suffering with erythroblastosis fetalis (Figure 4) Here the maternal anti Rh antibodies which had crossed the placenta have produced plateaus of approximately the same frequency height and receptor width (60 to 585 A) as those originally produced by adding anti Rh_0 antibody to Rh_0 positive erythrocytes in the test tube [7, 8] The receptor widths were again measured disregarding the inert surface between the reactive ends These surface distortions at the ends of the elevated plateaus were also of the same order as those defined by the Coombs reagent in Figure 3

Adherence of the antibody to a receptor site of the nature of Hillier and Hoffman s [3] erythrocytic surface plaque would comprise the structural basis for antigen antibody interaction in this system

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* We are indebted to Dr Joseph Eschbach for permission to study this case

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*In Vivo Localization of Specific Antiorgan Sera Relation to Occurrence of Renal Lesions**

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The results of administering specific antiorgan sera have been studied since Bordet[2] first reported that antibodies to rabbit blood when injected into rabbits were toxic. The initial damage produced by most antiorgan sera is rapidly repaired. In contrast, the injection of antikidney serum causes an acute glomerulonephritis which usually progresses to chronic nephritis ending in death from renal failure months or years later. Renal disease induced by this immunologic method has engaged the attention of many investigators because the pattern of human nephritis has been reproduced only by this means. Furthermore, the elucidation of the mechanism by which this disease perpetuates itself presents a challenge.

Previous studies have shown that the antigen or antigens in the kidneys which give rise to the nephritis producing or nephrotoxic antibodies are in high concentration in the glomeruli.[6, 11] There are two organs in addition to the kidney, the placenta[10] and the lung,[1, 12] which also stimulate the production of nephrotoxic antibody and these presumably possess antigen related to or identical with glomerular antigen. Nephrotoxic antibodies localize within the glomeruli, as has been shown by Pressman and his associates[8] by the injection of antikidney serum tagged with radioactive sulfur, and by Mellors and his associates[7] who have utilized the Coons technique to demonstrate the specific localization of rabbit anti*rat* kidney serum in glomerular basement membranes. These latter investigators also have reported the presence of rat globulin in the same basement membranes 6 or more days following the injection of the nephrotoxic serum.

The technique of Coons and Kaplan[3] has been employed in this laboratory to follow the distribution of specific nephrotoxic sera in rats, dogs and rabbits. The work has been done in association with Dr. Konrad Hsu and with the able assistance of Mrs. Mildred Rothenberg and Dr. Alfred

* This study was aided by Grant A 171 from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U.S. Public Health Service, and Grants G 2190 and G 4910 from the National Science Foundation.

Urquhart Antibodies to kidney lung and placenta have been produced by immunizing either the rabbit or duck These antisera have been injected into the specific hosts and the animals have been killed at intervals of 4 hours to 91 days the kidneys and other organs have been frozen sectioned in a cryostat, stained with fluorescein tagged antibody to the globulin of the species of animal supplying the nephrotoxic serum and

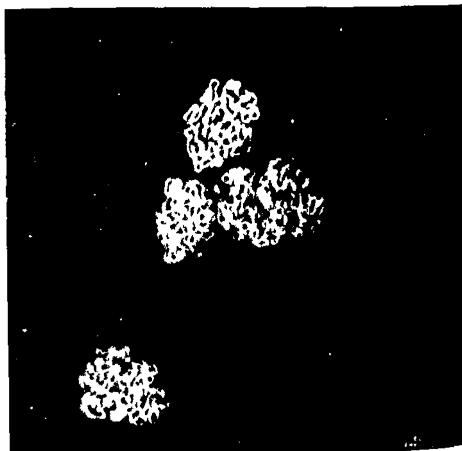


FIGURE 1 Section of kidney of rat SDC 56 killed 2 days after injection of 0.45 ml of rabbit anti*rat* kidney serum Stained with duck anti*rabbit* globulin tagged with fluorescein isocyanate Viewed in ultraviolet light Glomerular basement membranes fluoresce as they retain the antirabbit globulin ×250

examined microscopically in ultraviolet light Wherever the injected serum was retained in the tissues it bound the fluorescein tagged antibody and was identified by its fluorescence Control animals were injected with normal rabbit or duck serum and treated in the same manner as those receiving the nephrotoxic sera Some of the data gathered from these experiments are summarized

1 Nephrotoxic sera localize in the glomeruli of the kidney and largely in the glomerular basement membranes *in the rat* the rabbit and the dog Figure 1 is *from a* rat killed days after the injection of 0 45 ml of rabbit anti*rat kidney* serum The kidney section has been stained[1] with fluorescein tagged duck *antirabbit* globulin It may be seen that the nephrotoxic serum remains only in the glomeruli and apparently



FIGURE 2 A *higher magnification of the same kidney shown in Figure 1* One glomerulus is seen ×1000

particularly in the glomerular basement membranes as is better illustrated in Figure 2 which is a higher magnification of a glomerulus from the same section Figure 3 illustrates a section of *kidney* obtained by needle biopsy from a nephritic dog injected with rabbit anti*dog* placenta serum Again the injected serum is seen *only* in a glomerulus *of the* kidney The biopsy was taken 4 hours after *injection* Another biopsy 5 days later presented the same picture

2. Nephrotoxic sera remain in the glomeruli of the rat for at least 10 months. To date 9 animals have been killed after 100 to 291 days of nephritis and in every case the injected nephrotoxic serum has still been demonstrable in the glomeruli. Figure 4 shows the kidney of a rat 157 days after the injection of duck anti*rat* lung serum. By an immunologic

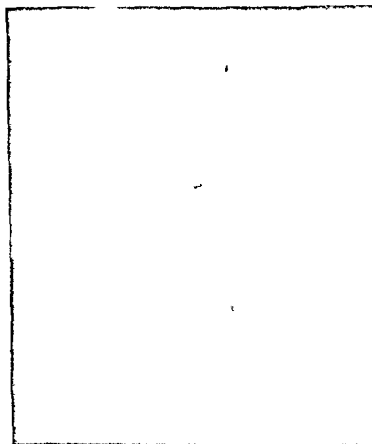


FIGURE 3. Section of kidney of dog Karen obtained by needle biopsy 4 hours after the last injection of rabbit anti*dog* placenta serum totaling ml per kilogram of body weight given on 3 successive days. Stained with fluorescein tagged duck anti*rabbit* globulin. The glomerulus is the only area in which the rabbit antiplacenta serum has localized. × 1000

method described elsewhere* it can be estimated that approximately 50 per cent of the original concentration of duck protein still remains in the glomerulus. In older rats this may be reduced to roughly 10 per cent.

3. The nephrotoxic sera have been demonstrated in extrarenal sites, particularly in cells of the red pulp of the spleen and in the cells lining

the capillaries of the adrenals The amount of nephrotoxic serum present in these areas is approximately 5 per cent to o per cent of that seen in the glomeruli In the rat the only animal so far studied over sufficient time it has been found that the nephrotoxic antiserum in the spleen and adrenal is greatly reduced at the end of 3 weeks and after 3 months it is

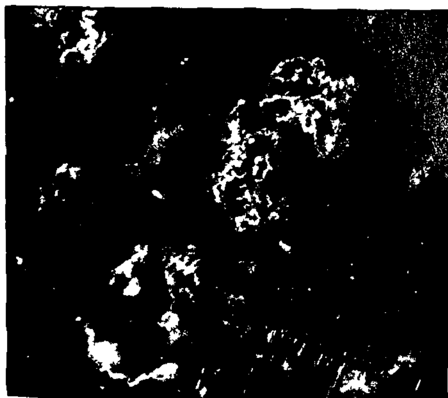


FIGURE 4 Section of kidney from rat SDC 7 killed 157 days after the injection of 1 5 ml of duck anti*rat* lung serum Stained with rabbit anti*duck* globulin tagged with fluorescein isocyanate Glomeruli still retain the injected duck serum ×500

demonstrable only in occasional cells Figure 5 is a section of adrenal from the same rat the kidney of which was shown in Figure 1 The fluorescent cells are apparently the cells lining the capillaries as determined by examination of contiguous sections stained with hemotoxylin and eosin Figure 6 illustrates a section of spleen from a rat injected 6 days previously with duck anti*rat* lung serum It can be seen that there are only a few fluorescent cells in the malpighian corpuscles but that many of the cells of the red pulp contain the injected antiserum

Evidence for the presence of the nephrotoxic sera in the liver has not been obtained and even antilung serum has not localized in the lung

4 There has been no localization in the glomeruli of normal rabbit or duck serum The localization of these sera in the spleen and adrenal has been less than that seen following the injection of the nephrotoxic sera

5 The final conclusion to be reported from these studies relates to

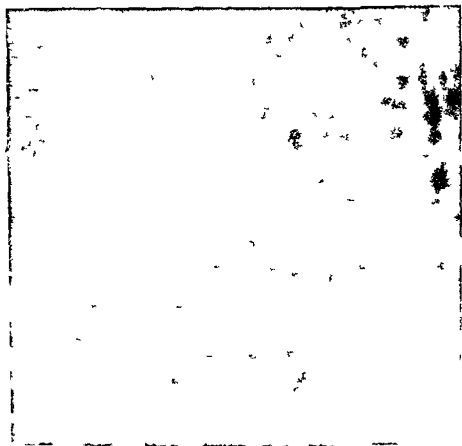


FIGURE 5 Section of adrenal of rat SDC 56 treated in the same manner as kidney section Figure 1 Cells which fluoresce are probably those lining capillaries × 1000

nephritis produced in the rabbit In order to present the results some introductory information must be given When an amount of nephrotoxic serum is injected which is below the threshold necessary to induce immediate evidence of renal damage nephritis nevertheless may develop after several days[4] This nephritis with a latent period is seen most frequently in rabbits injected with duck antirabbit kidney serum It was

suggested by Kay [5] that the delay in onset of the nephritis was due to the fact that the renal damage resulted from a reaction between antibodies produced by the host to the injected foreign nephrotoxic globulin and portions of this foreign protein specifically bound to the kidney This hypothesis to explain delayed nephritis puts the disease in the category of local serum sickness Unlike the serum nephritis which follows the



FIGURE 6 Section of spleen of rat SDB 817 killed 6 days after injection of 1 ml of duck anti*rat* lung serum Stained with fluorescein tagged rabbit anti*duck* globulin Scattered cells in red pulp fluoresce indicating the presence of the injected nephrotoxic serum × 250

injection of massive amounts of foreign serum or serum fractions and which is a part of the syndrome of serum disease the nephritis with a latent period that results from the injection of antikidney serum is a severe nephritis which does not usually heal and often terminates the life of the animal with renal failure months later

An attempt has been made by means of the Coons technique to investigate the possible role of 'local serum disease' in nephritis produced by duck anti*rabbit* kidney serum The serum was prepared by immunizing ducks with rabbit kidney over a period up to 2 months Unfortunately for the purposes of the experiment the serum proved to be so potent that when injected in rabbits even in small volumes proteinuria and

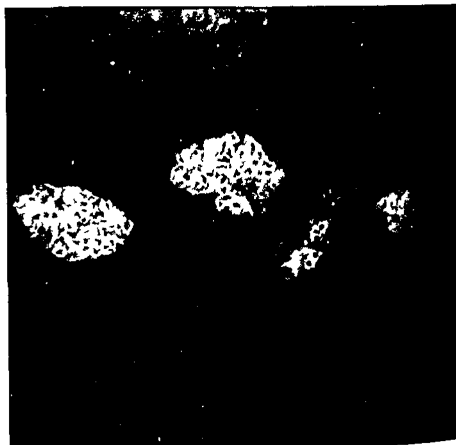


FIGURE 7 Section of kidney of rabbit 287 killed one day after the injection *of a total of 2 5 ml per kilogram of duck anti**rabbit** kidney serum* Stained with fluorescein tagged rabbit anti*duck* globulin The glomerular basement membranes contain the injected nephrotoxic serum as in the case of the rat and dog × 250

casts appeared in the urine immediately However the amount of proteinuria and the number of casts often increased about 6 days after injection and 4 of the animals died with renal failure at this time Sections of kidneys obtained from 1 to 14 days after injection have been stained with fluorescein tagged rabbit anti*duck* globulin and also with fluorescein

tagged duck anti*rabbit* globulin The purpose has been to determine if both the injected duck protein and rabbit globulin are concentrated in the glomeruli and at what time

Figure 7 shows a section of kidney from a rabbit which was injected with 5 ml per kilogram of duck anti*rabbit* kidney serum and killed the following day The section has been stained with tagged rabbit anti



FIGURE 8 Section of kidney from rat 186 killed 15 days after the injection of a total of 2 2 ml per kilogram of duck anti*rabbit* kidney serum Stained with fluorescein tagged rabbit anti*duck* globulin It is barely possible to see fluorescence in the glomerulus ×1000

duck globulin It may be seen that the nephrotoxic antiserum has localized in the glomeruli in a manner comparable to that demonstrated by other nephrotoxic sera injected in rats or dogs A section of the same kidney stained with duck anti*rabbit* globulin failed to demonstrate the presence of rabbit globulin in the glomeruli Examination of the kidney of a rabbit similarly injected with antikidney serum and killed 3 days later when

stained with rabbit anti*duck* globulin showed a somewhat decreased fluorescence in the glomeruli Staining of a second section of this kidney with duck anti*rabbit* globulin revealed fluorescence in cells lying between tubules and in some of the cells in the glomerular tufts which indicated the presence of rabbit globulin in these areas In other rabbits the kidney was examined 1 to 4 weeks after injection A section of a

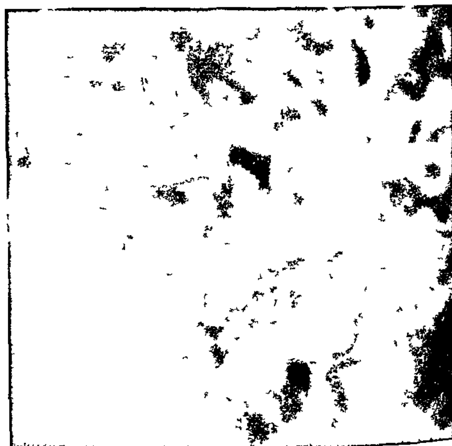


FIGURE 9 Section from the same kidney illustrated in Figure 8 stained with fluorescein tagged duck anti*rabbit* globulin The fluorescent antibody is bound in the glomerular basement membranes indicating the presence of rabbit globulin in this area ×1000

week kidney stained with rabbit anti*duck* globulin showed it was barely possible to demonstrate the injected duck protein in the glomeruli This is illustrated in Figure 8 which shows that fluorescence was very faint The reason for this was apparent when a section of this kidney was stained with tagged duck anti*rabbit* globulin Figure 9 shows the brilliant fluorescence in the glomeruli due to the presence of rabbit

globulin It appears that the nephrotoxic antibody which has localized in the glomerular basement membrane has been covered with antibody produced by the rabbit Since the duck antigen has already been bound to rabbit anti*duck* antibody it can no longer react with all the available tagged anti*duck* antibody This would account for the almost complete lack of fluorescence seen in the preceding figure

The results obtained in these experiments on nephritis in the rabbit support the theory of Kay It appears that rapid and plentiful production by the host of antibody to the foreign nephrotoxic serum results in an antigen antibody reaction in the glomeruli which can be looked upon as equivalent to local serum disease This reaction must certainly contribute to the damaging effect of the nephrotoxic serum

It may be concluded from the experimental data as a whole that the glomerular basement membrane possesses the property of retaining specific antibody in relatively large amounts and over a long period of time It is tempting to speculate that it is this unique property of the glomerulus which enables a single small injection of nephrotoxic serum to induce a self perpetuating disease fatal months or years later

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*Observations on the Site and Mechanisms of Antigen-Antibody Interaction in Anaphylactic Hypersensitivity**

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Previous work from this laboratory has been concerned primarily with the dynamics of antigen antibody interaction and the relationship of this process to the tissue lesions in hypersensitivity of the serum sickness type. These investigations integrated with the work of others may be briefly summarized.

Studies by Hawn and Janeway [1] and ourselves [2, 4] have demonstrated that the lesions of serum sickness appear as an animal begins to form antibody while antigen is still present in the circulation and tissue fluids. With continued production of antibody, antigen is eliminated, free antibody appears in the circulation and the organic manifestations of serum sickness resolve. Since antigen is present in the blood during the early antibody response, we suggested some years ago [3, 6] that the tissue lesions might arise secondarily to the transport of antibody by antigen in the form of soluble antigen antibody complexes. In the intervening years Sternberger *et al* [10] and Weigle [11] have demonstrated antigen antibody complexes in the circulation. Mellors *et al* [8] and Dixon *et al* [2] employing fluorescent antigen and antibodies found antigen and gamma globulin localized in the tissue lesions of hypersensitivity. It is not known whether the localization of gamma globulin in the tissues represents specifically fixed antibody. Other work from our laboratory has indicated that soluble antigen antibody complexes produced *in vitro* may elicit anaphylactic shock in guinea pigs, produce Arthus reactions in the skin and induce glomerular and splenic lesions of the serum sickness type following infusion into normal rabbits [4, 5]. Presumably these reactions require complement [9, 12].

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There is then considerable evidence that soluble antigen antibody complexes exhibit significant biologic activity and that they may play a leading role in the development of allergic reactions

It is clear that important strides have been made in an area which may be called the immunogenesis of immediate type allergic reactions Two of the most important problems which still demand clarification concern firstly the mechanism by which antigen antibody complement complexes injure tissue and secondly the site of tissue injury

In regard to the precise biochemical mechanism through which antigen antibody complement interaction results in tissue injury little is known Studies currently being carried on by others[1,10] on the role of complement in the hemolysis of red blood cells could provide data which might contribute to an understanding of tissue damage in allergic reactions Many workers believe that complement is concerned in the activation of an enzymatic system in the presence of reactive antigen and antibody[1,10]

In regard to the site of tissue injury in allergic reactions numerous studies have been made There seems little doubt that anaphylactic reactions are manifested in the vascular and connective tissue systems Whether damage to the vascular system is primarily an expression of alteration in the connective tissue which is indissolubly associated with blood vessels or whether these changes are due to endothelial injury has never been resolved Since marked alteration of the connective tissues is prominent in hypersensitivity the first of these possibilities seems more acceptable and warrants further investigation

Some years ago Rich and Follis[11] failed to produce a typical hemorrhagic Arthus reaction in the rabbit cornea unless vascularization had been previously induced They noted slight edema slight polymorphonuclear infiltration and moderate swelling of the corneal fibers 4 and 48 hours after injection of horse serum into the avascular cornea of sensitized animals It would seem that these workers produced a true Arthus phenomenon in the vascularized corneas of sensitized animals However this might be expected under the conditions of the experiment since vascularization of the cornea in effect converts the cornea into a structure very similar to skin

Since the cornea consists of almost pure connective tissue comprised of fibroblasts collagen and reticulum fibers and amorphous grand substance far removed from a blood supply and since there is so much experimental and pathologic evidence of connective tissue damage in hypersensitivity it was deemed useful to employ this tissue in further studies of hypersensitivity using quantitative immunologic methods to see whether connective tissue damage could indeed be related to antigen antibody interaction in the absence of blood vessels Recent work in our laboratory has disclosed that connective tissue change can be produced

in the avascular cornea at the site of local antigen antibody interaction These experiments were of two designs In one type appropriate quantities of bovine serum albumin or bovine serum gamma globulin were injected into the avascular cornea of passively or actively sensitized rabbits In the second type of experiment bovine serum albumin and its corresponding antibody were simultaneously injected into different sites of the avascular corneas of normal rabbits In all experiments alterations in the connective tissue were observed which have been interpreted as those of degeneration or necrosis Although these changes were accompanied by acute exudative inflammation they did not appear to be dependent on leukocyte migration since similar changes were noted in animals which had been rendered leukopenic by nitrogen mustard injections In each case the connective tissue alteration occurred at the site of maximal precipitation of antigen antibody complexes as demonstrated by fluor marking of the injected antigen

Following the injection of 0 1 ml of fluor labeled foreign protein into the center of the cornea of actively or passively sensitized animals the limbus appeared grossly inflamed 6 to 8 hours later From 1 to 4 hours later a grayish arc of opacification appeared in the periphery of the cornea It was separated from the limbus by a clear area 1 to 3 mm in width (Figure 1) Further observation for an additional 4 hours generally disclosed completion of the arc to form a concentric ring of opacification Examination of such eyes under Wood s light in the living animal revealed sharp localization of antigen in the ring of opacification Histologic examination of corneas showing such rings (Figure) revealed a sharp line of concentrated leukocytes traversing the cornea from Bowman s to Descemet s membrane The leukocytes had evidently migrated from the limbal side as evidenced by the presence of scattered leukocytes between the limbus and the sharp line of leukocytic infiltration The limbal vessels were dilated and engorged and the loose connective tissue around them was infiltrated by leukocytes red blood cells and fibrin Closer examination of the corneal line of leukocytes disclosed fragmentation of deeply eosinophilic swollen collagen fibers The surrounding cells appeared pyknotic and their differentiation was not possible (Figure 3) When corneal sections were examined with ultraviolet illumination the following typical changes were noted (Figure 4) There was maximal fluorescence along the corneal line of most intense leukocyte concentration and collagen fragmentation No fluorescence was evident beyond this line toward the limbus except for autofluorescence of the granular leukocytes The corneal tissue between the site of injection and the line showed diffuse pale greenish fluorescence indicative of antigen diffusing from the site of injection toward the periphery of the cornea No blood vessels were found in the cornea

FIGURE 1 Living eye in rabbit previously sensitized to bovine albumin Twenty four hours after injection of 1 mg fluor-bovine albumin into center of cornea Note *opaque* ring in periphery of cornea with clear cornea between ring and limbus Dilatation and engorgement of limbal and bulbar conjunctival vessels with petechial hemorrhages Under Wood's light the gray opaque ring presented bright green fluorescence (The two bright spots are highlights)



FIGURE 2 Transverse section of peripheral portion of cornea and adjacent limbus Rabbit previously sensitized with bovine albumin Received injection of 1 mg fluor bovine serum albumin in center of cornea 30 hours before sacrifice Hemorrhage and neutrophilic leukocytic infiltration around limbal vascular loops Sharp transverse line of leukocytic infiltration and collagen degeneration distant from limbus This line corresponds to a section through the opaque ring seen grossly Note leukocytes migrating from limbus across clear corneal zone to line No blood vessels are present in cornea

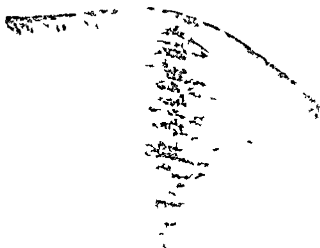




FIGURE 4 Fluorescence preparation of lesion similar to that of Figure 3 The intense white line represents a bright green line of fluorescence under ultraviolet illumination due to fixation of fluor-antigen by antibody diffusing from limbus in a sensitized animal 30 hours after intracorneal injection of 1 mg fluor bovine serum albumin The line of fluorescence coincides precisely with the line of leukocyte infiltration and collagen degeneration (The bright appearance of the cells is due to auto fluorescence)

antibody precipitation in the corneas of nonsensitized animals was followed grossly over a period of days following the simultaneous injection of antigen and antibody it was observed that the line progressed as an opaque front away from the antigen side toward the antibody side This was readily demonstrable by marking the cornea at the initial line of precipitation with India ink Progressive development of the zone of opacification could be equated with antigen antibody interaction as

evidenced by gross examination under Wood's light and by fluorescence microscopy

It is believed that these experiments demonstrate that antigen antibody interaction can produce immediate damage to the connective tissue stroma of the avascular rabbit cornea and that this damage is exquisitely localized at the site of interaction. Study of such lesions uncomplicated by the confusing effects and local participation of blood vessels as in the hemorrhagic Arthus phenomenon in the skin has obvious advantage not only for morphologic analysis but more importantly for chemical and enzymatic investigation

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II

*Role of in Vivo Antigen–Antibody Precipitation in Hypersensitivity Reactions**

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Obstruction of the pulmonary circulation in rabbits undergoing anaphylactic shock is usually attributed to spasm of the pulmonary vascular bed[1 2 4] but in several histologic studies there have been described amorphous eosinophilic masses in distended pulmonary capillaries[6 12 1]. By most these were considered to be thrombi resulting from focal capillary damage produced by the antigen antibody reaction. Several observations however suggest that these so called thrombi may be *in vivo* precipitates of antigen and antibody.

Firstly, high levels of circulating precipitating antibody are required to elicit anaphylactic shock in the rabbit. Secondly, in previous studies on experimental serum sickness hyaline thrombi were often observed in the pulmonary capillaries and arterioles of rabbits receiving *repeated* injections of bovine albumin or bovine gamma globulin. When these rabbits showed signs of anaphylactic shock pulmonary thrombi were invariably present. On the other hand pulmonary thrombi of this type were never encountered in rabbits developing serum sickness after a *single* large injection of antigen where sudden interaction of large amounts of antibody and antigen is unlikely. Thirdly, in a report on the use of I labeled antigens in immunologic investigation Dixon described concentrations of radioactivity in eosinophilic masses occluding the pulmonary capillaries of rabbits subjected to anaphylactic shock. He suggested that the intracapillary masses might be I^{131} antigen antibody precipitates and that obstruction by these masses might play an important role in anaphylactic shock in the rabbit.[8]

The experimental methods and results of our work undertaken with the intention of testing this supposition have been recorded in detail elsewhere.[11] In brief, rabbits were sensitized to bovine serum albumin (BSA)

* This work was performed in collaboration with Drs F C Andrews, R H Heptinstall and F G Germuth of the Department of Pathology, The Johns Hopkins University School of Medicine and was supported in part by Grant A 715 from the U S Public Health Service to Dr Germuth

or to bovine gamma globulin (BGG) After high antibody titers had developed these sensitized animals together with normal control animals were challenged intravenously with the respective antigens made visibly detectable by conjugation with fluorescein (Fluor BSA Fluor BGG)

All of the sensitized animals so treated showed signs of anaphylactic shock Those which had not died were killed at the end of 10 minutes Tissues from the entire group were prepared for histologic examination of stained sections and by fluorescence microscopy

As shown in Table I sensitized rabbits shocked with the homologous

TABLE I

Rabbit Sensitized to	Injected with	Masses in Pulmonary Capillaries	Green Fluorescence of Masses
BSA	Fluor BSA	+	+
BSA	BSA and Fluor BGG	+	—
BGG	Fluor BGG	+	+
BGG	BGG and Fluor BSA	+	—
Normal	Fluor BSA or Fluor BGG	—	—

antigen showed amorphous eosinophilic masses in distended pulmonary capillaries If the homologous antigen was fluorescein labeled a sharply localized bright yellow green fluorescence could be seen in such masses under ultraviolet illumination but if the homologous antigen was un labeled and injected in a mixture with fluorescein labeled heterologous protein no such fluorescence appeared in the intracapillary masses Neither masses nor localized foci of fluorescence were found in the lungs of normal unsensitized animals injected with these fluorescein labeled antigens

The specific localization of antigen in the pulmonary thrombi in sensitized animals strongly suggested that they were antigen antibody precipitates Subsequently normal rabbits were injected intravenously with suspensions of washed immune precipitates made *in vitro* whereupon the normal animals exhibited signs and anatomic changes indistinguishable from those seen in ordinary anaphylactic shock in sensitized rabbits

Since nystagmus weaving of the head and a peculiar circling move ment are often seen during anaphylactic shock in the rabbit sections of cerebellum were examined for vascular occlusions In the tiny capillaries of the brain precipitates were not easily identified in the hematoxylin and eosin stained sections but under ultraviolet illumination the yellow green fluorescence of antigen in such masses readily distinguished them from red cells or plasma in a capillary

Precipitates were similarly seen in the capillaries of the renal glomeruli, in splenic sinusoids at the periphery of the malpighian bodies, and in the liver. In the liver they were sometimes quite large, filling the terminal portal veins and extending into the sinusoids.

The occurrence of precipitates in the portal veins and hepatic sinusoids of rabbits undergoing anaphylactic shock suggested a possible explanation for the old and puzzling observation of "focal allergic necrosis of the liver" following repeated injections of foreign protein.[9] Accordingly, sensitized rabbits were challenged by the injection of fluorescein-labeled antigens directly into the portal circulation. In hematoxylin and eosin sections of the livers typical eosinophilic masses were seen in the portal veins and adjacent sinusoids. These "thrombi" showed bright yellow-green fluorescence only if the fluorescein-labeled protein was that to which the animal had been sensitized. No parenchymal alterations were present in animals killed or dying within 24 hours after injection, but in animals surviving for 24 hours many portal and midzonal hepatic necroses were seen. By this time the precipitates and the necrotic areas were infiltrated with polymorphonuclear leukocytes. When the precipitates in the portal end of the sinusoids and the necrotic foci lay in the same plane of the section, a most convincing visual impression was gained that obstruction of the vascular channels by precipitates had led to infarction of the liver cells.

In addition, normal animals were injected by the portal vein route with suspensions of washed immune precipitates made *in vitro*. In the livers of these animals sacrificed 24 hours after injection there were amorphous intravascular masses identical in appearance and distribution with those seen in the sensitized animals. Local areas of hepatic necrosis were also observed in the portal zones.

The portal necroses produced in these animals are quite similar to those described after embolization of the terminal portal veins by lycopodium spores and other particulate matter.

The foregoing observations suggest an explanation for some of the unusual aspects of anaphylaxis in the rabbit and should be considered when comparisons are made with the reactions of hypersensitivity in man and other animals where *in vivo* antigen-antibody precipitation has not been demonstrated.

A

B

FIGURE 1 Rabbit sensitized to BSA injected with fluorescein labeled BSA (A) Lung showing eosinophilic intracapillary precipitates H & E ×300 (B) Same microscopic field under ultraviolet irradiation showing fluorescence of precipitates

Reproduction of illustrations by courtesy of the *Bulletin of The Johns Hopkins Hospital*

A

B

FIGURE 2 Rabbit sensitized to BSA injected with unlabeled BSA and fluorescein labeled BGG (A) Lung showing intracapillary precipitates H & E ×300 (B) Same field under ultraviolet irradiation showing lack of fluorescence of precipitates

A

B

FIGURE 3. Rabbit sensitized to PSA injected with fluorescein labeled PSA. (A) Brain showing precipitate in tiny capillaries. H & E. ×300. (B) Same field under ultraviolet irradiation showing fluorescence of precipitates.

A

B

FIGURE 4 Rabbit sensitized to BSA injected with fluorescein labeled BSA (A) Liver showing precipitates in portal vein and extending into sinusoids H & E ×300 (B) Same field under ultraviolet showing fluorescence of precipitates Note absence of fluorescence of red blood cells in same vessel

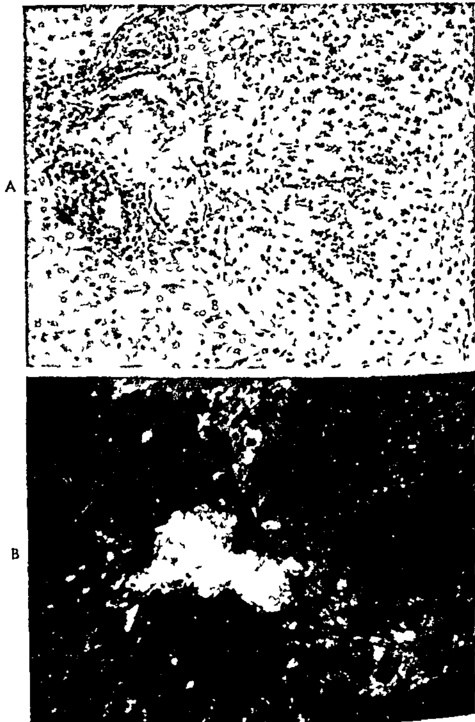


FIGURE 5 Rabbit sensitized to BSA injected by way of mesenteric vein with fluorescein labeled BSA Animal sacrificed 4 hours after injection (A) Liver showing precipitates in portal vein and infarct like necrosis of adjacent liver cells Note leukocytic infiltration of precipitate and of necrotic area H & E ×170 (B) Same field under ultraviolet irradiation

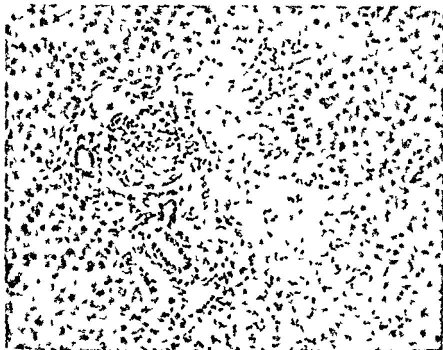


FIGURE 6 Normal rabbit injected by way of mesenteric vein with suspension of washed antigen antibody precipitate made *in vitro* Animal sacrificed 24 hours after injection Note infarct like necrosis and leukocytic infiltration of precipitate and necrotic area H & E ×170

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12

*Studies on Nonprecipitating Antibody III An Antigen-like Material Obtained from Serum after Disappearance of the Original Antigen**

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The occurrence of precipitates in alkali treated immune sera is puzzling in several ways The sequence of appearance of this material as well as its solubility in very large excesses of specific antigen suggests that it may be an antibody antigen complex[17] However certain differences exist between such a complex and the complexes obtained when antigen is reacted with precipitating antibody *in vitro* One involves its solubility the material obtained in relatively large amounts by alkali treatment *in vitro* is derived from a soluble source in serum Large amounts of precipitating antibody and antigen cannot coexist in this form except in antigen excess as found early in immunization[20] The other difference involves the nature of any antigen component of the complex It is known from the studies of Dixon and his group[6,19] that the original antigen injected disappears rapidly from circulation in response to the formation of antibody The material obtained by alkali treatment reaches a peak however when the injected antigen is eliminated from circulation and precipitating antibody is first detected and it persists in circulation beyond the time during which the original antigen can be found Also Weigle[20] has shown that the precipitates obtained by alkali treatment following injection of I^{131} labeled antigen contain only traces of radioactivity

Attempts to study these differences have disclosed one component of the alkali precipitates previously[16] Another factor will be reported in the present paper The first component was shown to be a nonprecipitating antibody which upon alkali treatment is converted to a precipitin like material This nonprecipitating antibody appeared in two phases following alkali treatment One fraction participates in the spontaneous precipitates

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which occur upon alkali treatment without addition of antigen The remainder is retained in solution but will precipitate upon addition of specific antigen but not upon addition of nonspecific protein The dissociability of the products of the latter reaction is greater than that of precipitating antibody and antigen Antigen may coexist with antibody *in the supernates and larger amounts of antigen are needed for inhibition* It thus appears that this nonprecipitating antibody reacts with the original antigen in a manner similar to the behavior of a cross reaction antibody [14] Does this suggest the possibility of the existence of an antigen which is a more specific or so to say more natural substrate to this antibody If nonprecipitating antibody would react with such an antigen *in vivo*, it could explain why after alkali treatment some of the nonprecipitating antibody precipitates spontaneously, while another fraction remains in solution Accordingly we have now carried out immunization studies with the precipitates obtained after alkali treatment to disclose the presence of any masked antigenic factor

Methods

Obtainment of Complex from Donor Rabbits

New Zealand white male rabbits bred at the Army Chemical Center 6 months old and weighing about 3 Kg were given native or I^{131} labeled BPG (bovine plasma globulin Pentex Inc) intravenously either in a single injection in amounts of 1 Gm /Kg or in repeated biweekly injections in amounts of 0 1 Gm /Kg Radioiodination was carried out by the method of Pressman and Eisen [15] Rabbits receiving labeled antigen were *given about 3 mg of potassium iodide daily in their drinking* water for one week before injection Sera were obtained on the thirteenth and fifteenth days after the 1 Gm /Kg dose and on the eighth day after repeated 0 1 Gm /Kg doses

The individual sera were tested for the presence of injected antigen by radioactivity determinations and by precipitating with antiserum In order to detect small amounts of I^{131} the ratio of counts to background was reduced by the use of a well type scintillation counter with 3 inches of lead shielding and a spectrum analyzer amplifier with the gain so adjusted that the 0 608 Mev gamma emission of I^{131} would give a 56 5 volt signal in the analyzer Signals of window width between 46 5 and 66 5 volts were registered Under these conditions 1 millicurie of iodine gave 4×10^{8} counts per minute against a background averaging 6 counts per minute For precipitation with antiserum 0 ml of sera were mixed with 0 ml of anti BPG produced with Freund s complete adjuvants [7] The technique and proportions appear in a previous publication [16] The tubes were incubated at 37° C for one hour and left at 1° C overnight and precipitates if any estimated qualitatively

The individual sera were also tested for the presence of precipitating antibody by admixture of 0 ml with equal volumes of 1 40 000 and 1 4000 BPG Precipitates were estimated qualitatively following incubation at 37 C for one hour and storage at 1 C overnight

The sera which were free of antigen and contained excess of free precipitating antibody were pooled brought to pH 1 6 at 1 C for 6 minutes neutralized and left at 1 C for 11 days as described previously[16] The precipitates were washed three times at 1 C with 0 16 N sodium chloride solution (400 ml for the first washing 00 ml for the second washing and 60 ml for the third washing) Portions of the precipitates were counted for radioactivity when applicable The remaining precipitates were resuspended in 0 16 N sodium chloride solution by drawing through a pipette 0 times Sufficient solution was used to yield suspensions containing either 7 5 or 15 0 mg N ml Part of these suspensions was used immediately and the remainder was frozen at —60 C and stored at — 0° C for 1 to 3 weeks

Immunization of Recipient Rabbits

New Zealand white male rabbits bred at the Army Chemical Center 6 months old and weighing about 3 kg were given native or I^{131}-labeled material obtained by alkali treatment from 3 different serum pools (Complex I II and III in Table I) The rabbits receiving complex I and II were given the following series of 4 simultaneous intramuscular injections first series 9 mg N in Freund s complete adjuvants in the proportions used in the method of Sternberger and Pressman[1] second series 9 mg N suspended in 0 16 N sodium chloride solution 1 days later The rabbits receiving complex III were given series of 4 simultaneous intramuscular injections by the following schedule first series 14 mg N in Freund s complete adjuvants second series 11 mg N in Freund s incomplete adjuvants (omitting the tubercle bacilli) 7 days later third series 15 mg N in 0 16 N sodium chloride solution 7 days after the second series (On the average each rabbit received complex isolated from 135 ml of serum) All animals were bled on the twenty ninth day after the first injection and each subsequent 7 days Merthiolate[(1)] 1 10 000 was added and the sera were stored at 1 C for the series of tests including bleedings up to the forty third day or they were frozen at —60° C and stored at — 0° C for the series of tests including bleedings up to the seventy first day

Control Immune Sera

Cockroach hemolymph Aedes aegypti and pleuropneumonia like organisms were used as control antigens that do not cross react with PPG Cockroach hemolymph was obtained from the anterior heart following pretreatment of cultured roaches (*Blatella germanica*) with tissue

TABLE I PRESENCE OF INJECTED ANTIGEN AND OF ANTIBODY IN SERA FROM DONOR RABBITS

Sera	Presence of Injected Antigen		Presence of Precipitating Antibody	
	Per Cent Radioactivity Injected	Precipitation with Antiserum	Precipitation with 1 40 000 BPG	Precipitation with 1 4000 BPG
Sera accepted for pool of Complex I				
6 individual sera obtained 13 days after 1 Gm /Kg BPG i v		–	+†	++‡
6 individual sera obtained 15 days after 1 Gm /Kg BPG i v		–	+	++
6 individual sera obtained 8 days after a second injection of BPG (0 1 Gm /Kg)		–	+	++
Sera accepted for pool of Complex II				
Sera obtained 13 days after 1 Gm /Kg BPG i v				
Rabbit No 1		–	+	++
Rabbit No 2		–	+	++
Sera obtained 15 days after 1 Gm /Kg BPG i v				
Rabbit No 1		–	+	+
Rabbit No 2		–	+	++
Sera obtained 7 days after repeated injections of 0 1 Gm /Kg BPG i v every 14 days				
Rabbit No 3 after				
3 injec				
4 injec		–	+	++
5 injec		–	+	++
6 injec		–	+	++
		–	+	++

Sera	I er C nt Radio activity Injected	Prec p ta tion with Antiserum	P ec p ta tion with 1 40 000 BI C	I recipita tion w th 1 4000 BPC
Sera accepted for poo l of Complex III				
Sera obtained 13 days after 1 Gm/kg BPG i v				
Rabbit No 1	0 04	–	+	++
Rabbit No 2	0 03	–	+	++
Rabbit No 4	0 03	–	+	++
Rabbit No 5	0 01	–	+	++
Rabbit No 6	0 01	–	+	++
Rabbit No 7	0 0?	–	+	++
Rabbit No 8	0 01	–	+	++
Sera obtained 15 days after 1 Gm/kg BPG i v				
Rabbit No 1	0 0?	–	+	++
Rabbit No ?	0 00	–	+	++
Rabbit No 3	0 01	–	+	++
Rabbit No 4	0 05	–	+	++
Rabbit No 5	0 08	–	+	++
Rabbit No 8	0 01	–	+	++
Sera rejected for pool of Complex III				
Serum obtained 13 days after 1 Gm/kg BPG i v				
Rabbit No 3	0 01	–	±§	++
Serum obtained 15 days after 1 Gm/kg BPG i v				
Rabbit No 6	0.0	–	–	+
Rabbit No	0 45	–	±	+

No one of the sera examined in this group were rejected
+ Moderate precipitate
Heavy precipitate
§ Faint precipitate

juice from the same species This was found to render cockroach hemolymph incoagulable Aedes aegypti strain Germany, was obtained from Dr George Craig Pleuropneumonia like organisms were isolated from rats and grown in thioglycollate broth with added 5 per cent dog ascitic fluid Rabbits were immunized with 80 mg N of either hemolymph or homogenized Aedes aegypti or with one million living and appreciably more dead pleuropneumonia like organisms incorporated in Freund s complete adjuvants The anti cockroach hemolymph and anti Aedes aegypti sera used contained more than 500 μg of specific antibody N per milliliter

Testing of Sera Produced in Recipient Rabbits

Estimation of antibodies was carried out by the tannic acid hemoagglutination test of Boyden as described by Heller and co workers[4] Most sera were preabsorbed with an equal volume of a 33½ per cent suspension of washed sheep cells under constant shaking in a 37° C water bath In each experiment tannic acid treated cells were sensitized with 1 1000 BPG 1 1000 HPA * and 0 16 N sodium chloride solution each containing 1 10 000 Merthiolate[(P)] In some experiments other concentrations of BPG and HPA as well as cells not treated with tannic acid were also included The sera were diluted in phosphate buffer[9] (containing 1 per cent absorbed normal rabbit serum) by a factor of starting with a 1 50 dilution Agglutinations were set up on the same day the cells were sensitized Inhibition tests were carried out the following day by adding to 0 5 ml of each serum dilution 0 25 ml of 1 1000 BPG 0 5 ml of 1 1000 HPA and 0 5 ml of 0 16 N sodium chloride solution respectively each containing 1 10 000 Merthiolate[(P)] After incubation at 37° C for ½ hour the tubes were admixed with 0 5 ml of tannic acid treated cells sensitized with 1 1000 BPG which had been prepared on the previous day and had been stored at room temperature overnight Agglutination inhibition was read after leaving the tubes at 1° C overnight

Precipitin tests were carried out by the method of Heidelberger and Kendall[8] in the manner described in reference 17 Antibody was estimated from the precipitates obtained at equivalence

Results

Three serum pools were used in this study The complex isolated from the first pool was injected into 4 rabbits and that from the second and third pools into 2 rabbits None of the sera obtained from the donor rabbits for pools I and II contained free antigen by precipitation tests and they all contained a fair excess of free precipitating antibody (Table I) Three of the sera from the donor rabbits from pool III how

Human Plasma Albumin obtained through the courtesy of Dr Benjamin F Sanders Merck Institute for Therapeutic Research West Point Pennsylvania

ever were rejected — one of them because of its contents of radioactivity and all three of them because of the presence of only small amounts of free antibody. The sera accepted for pool III contained low and inconsistent levels of radioactivity on the thirteenth and fifteenth days after injection. The low levels of radioactivity obtained during this period seem to show irregular daily variations and may represent in large part material incorporated in thyroid products.[5]

The complex isolated from pool III was free of radioactivity (total counts of background 1 065, 11 605, 11 6 9 and 11 06 on four consecutive days; corresponding counts of sample plus background 1 11, 11 640, 11 634 and 11 5 5).

A typical hemoagglutination test with serum from recipient rabbits is shown in Figure 1. No agglutination was observed with untreated cells,

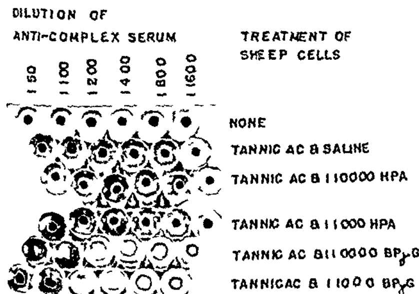


FIGURE 1. Patterns of agglutination of anticomplex serum and sensitized tannic acid treated sheep erythrocytes

with cells treated with tannic acid but not sensitized and with treated cells sensitized with 1 10 000 and 1 1000 HPA. With cells sensitized with 1 10 000 or 1 1000 BPC agglutination occurred to a titer of 1 800. Table II lists the results of titrations of sera obtained 29 to 71 days after immunization of 8 rabbits with any of the 3 complexes used. Seven of these rabbits showed anti BPC antibodies from the twenty ninth to the forty third day in titers from 1 50 to 1 3200. One of 3 rabbits lost its titer by the sixty fourth day but in the other 2 rabbits tested late after im

TABLE II AGGLUTINATION OF SENSITIZED SHEEP CELLS BY ANTICOMPLEX SERUM

Antiserum	Serum No	Day of Bleeding‡	Hemoagglutination Titer BPG	HPA	MNSS	Inhibition BPG†	HPA†
Anti complex I	A	43	1 100	–	–	+	–
	B	43	1 200	–	–		
	C	36	1 400	–	–	+	–
		43	1 800	–	–	+	–
		64	1 400	–	–	+	–
	D	43	1 50	–	–		
Anti complex II	E	36	1 200	–	–	+	–
		4	1 100	–		+	–
		71	1 100		–	+	–
	F	36	1 400	–		+	–
		4[illegible]	1 400	1 50	–		
		64	–	–	–		
		71	–	–	–		
Anti complex III	G	[illegible]	–	–	–		
		36		–	–		
		43	–	–	–		
	H	9	1 3200	–	–	+	–
		36	1 3200	–	–	+	–
		43	1 1600	–		+	–
Anti-cockroach hemolymph	J	54	–	–	–		
Anti Aedes aegypti	K	35	–	–	–		
		4[illegible]	–	–	–		
	L	4[illegible]		–	–		
Anti pleuropneumonia like *organisms*	M	35	–	–	–		
Normal serum	N			–	–		
	O			–	–		
	P		1 1600	1 50	1 50	+	+
	Q			–	–		
	R		–	–	–		
	S		–	–	–		
	T		–	–	–		
	U		–	–	–		
	V		–	–	–		
	W		–	–	–		
	X		–	–	–		
	Y		–	–	–		

Antigen used for sensitization of tannic acid treated sheep cells MNSS 0 16 N s chloride solution containing 1 10 000 Merthiolate( )

† Antigen used for inhibition of agglutination with BPG sensitized tannic acid treated cells

‡ After first immunization injection

munization antibody persisted until the sixty fourth and seventy first days respectively Rabbit H discussed below, also showed persistence of antibody until at least the seventy seventh day Reaction with the nonspecific antigen HPA occurred in only 1 rabbit at a low titer of 1 50 None of the anticomplex sera reacted with nonsensitized tannic acid treated cells

Inhibition reactions were either positive or negative and never partial — that is when inhibition was positive agglutination was absent in any of the serum dilutions tested when it was negative agglutination was present to the same titer as in the noninhibited agglutination tests The agglutination reactions of all the anticomplex sera which were tested for inhibition were abolished by BPG but not by HPA Unfortunately no inhibition tests were carried out with the anticomplex serum which gave a 1 50 agglutination reaction with HPA sensitized cells

The anti cockroach hemolymph the anti Aedes aegypti and the anti pleuropneumonia like organisms sera and 1 out of 13 normal sera failed to react with either BPG or HPA, or nonsensitized tannic acid treated cells One normal serum however showed a titer of 1 1600 with BPG sensitized cells and a titer of 1 50 with either HPA sensitized or unsensitized tannic acid treated cells This seems to be a nonspecific reaction because it was inhibited by both BPG and HPA

The amounts of antibody formed in the anticomplex sera were too small for precipitin analysis except in the case of serum H from immunization with complex III (Table III) The amount of antibody in this serum was 30 μg N/ml on the twenty ninth day after immunization 4 μg N/ml on the thirty sixth day 25 μg N/ml on the forty third day 17 μg N/ml on the fiftieth day and 14 μg N/ml on the seventy seventh day Comparison of these values for the best anticomplex serum with those for our best anti BPG sera which contain 3000 μg N/ml indicates that immunization by complex is only about one hundredth as efficient as direct immunization with BPG Serum H did not contain nonprecipitating antibody disclosed by alkali treatment [14]

Analysis of the supernates (Table III) offered opportunity to compare the properties of the antibody formed after immunization with complex with those of nonprecipitating antibody converted to a precipitin like material In the case of serum H there was a clear antigen and antibody excess zone Antibody did not coexist with antigen in the same supernates as in the case of nonprecipitating antibody converted to a precipitin like material [16] The maximum was close to the equivalence zone in the present serum while in the case of nonprecipitating antibody converted to a precipitin like material the maximum is in the antigen excess zone Finally the reaction was completely inhibited by 416 γ g BPG N With converted nonprecipitating antibody much larger amounts of antigen are necessary for complete inhibition [1] The antibody in serum H resembles therefore

TABLE III Precipitation of Anticomplex Serum H with BPG

Day After First Injection	Added µg BPG N/ml Serum	Precipitates		Supernates	
		µg N/ml Serum	µg Antibody N/ml Serum	Excess Antigen	Excess Antibody
29	4	34	30	−	−
	8	38		+	−
	17	36		+	−
	42	40		+	−
	167	28		+	−
	416	0		+	−
36	4	30	26	−	+
	8	50	42	−	−
	1	58		+	−
	42	53		+	−
	83	50		+	−
	167	38		+	−
	416	0		+	−
	832	0		+	−
43	4	9	25		−
	8	29		+	−
	17	27		+	−
	21	29		+	−
	42	trace		+	−
	83	trace		+	−
	16	0		+	−
	208	0		+	−
	416	0		+	−
50	4	1	17	−	−
	8	17		+	−
	17	trace		+	−
	1	0		+	−
	42	0		+	−
	83	0		+	−
	167	0		+	−
	208	0		+	−
	416	0		+	−
77	4	17	14	−	−
	8	trace		+	
	17	0		+	
	21	0		+	−
	4	0		+	−
	83	0		+	−
	167	0		+	−
	208	0		+	−
	416	0		+	−

* Average of duplicate determinations

precipitating antibody and not nonprecipitating antibody converted to a precipitin like material

In order to compare the properties of the antibody formed after immunization with complex and those of antibody formed after direct immunization with BPC the reactions of both types of antisera with cells sensitized with varying amounts of BPG were evaluated (Table IV) With one anticomplex serum optimal agglutination occurred with cells sensitized with 1 100 to 1 000 BPG with the other the reaction was optimal with cells sensitized with 1 000 BPG In the case of a pooled anti BPG produced with Freund s complete adjuvants and containing 5/8 μg N/ml the reaction was optimal with cells sensitized with 1 000 BPG The ranges for optimal sensitization of sheep cells in the cases of anticomplex serum and anti BPG are therefore comparable

In another experiment the concentrations of BPG required to inhibit partially the reaction of anticomplex serum and cells sensitized with 1 1000 bPG were compared with the concentrations required for similar degrees of inhibition of the reaction of the pooled anti BPG and sensitized cells Table V shows that similar amounts of BPG were needed to reduce the titer to one fourth and one eighth in the case of anticomplex serum F For reduction of the titer of anticomplex serum F to one half about 10 tim s as much BPG was needed as was required for a similar reduction in titer of anti BPG Similar degrees of inhibition were accomplished with anticomplex serum C by slightly less antigen than with anti BPG

COMMENTS

Following the disappearance of injected BPG from donor rabbits their sera were treated by alkali and the ensuing precipitates transferred into recipient rabbits The sera of the recipient rabbits showed the presence

TABLE IV CONCENTRATION OF BPG REQUIRED FOR OPTICAL SENSITIZATION OF TANNIC ACID TREATED CELLS IN THE AGGLUTINATION BY ANTICOMPLEX AND ANTI BPG SERUM

Antiserum	Concentration of BPG Used in Coating Cells						
	1 20	1 100	1 400	1 000	1 10 000	1 40 000	1 200 000
	Agglutination Titer						
Anticomplex serum B 43 days bleeding	1 50	1 00	1 200	1 200	1 50	—	—
Anticomplex serum C 36 days bleeding	1 50	1 200	1 200	1 400	1 100	—	—
Pooled anti BPG	1 50 000	1 100 000	1 100 000	1 200 000	1 50 000	1 12 500	1 1 500

TABLE V CONCENTRATION OF BPG REQUIRED FOR PARTIAL INHIBITION OF AGGLUTINATION BY ANTICOMPLEX AND ANTI BPG SERUM

Antiserum	Reduction of Titer		
	½	¼	⅛
	Concentration of BPG Required		
Anticomplex serum C 43 days bleeding	1:100 000 000	1:4 000 000	1:1 000 000
Anticomplex serum F 36 days bleeding	1:4 000 000	1:1 000 000	1:400 000
Anti BPG	1:40 000 000	1:1 000 000	1:400 000
	Ratio of Concentration of BPG Inhibiting Anticomplex Serum to That Inhibiting Anti BPG		
Anticomplex serum C	0.4	0.25	0.4
Anticomplex serum F	10	1	1

of anti BPG on subsequent examination Such antibody could be derived from several sources (*a*) it could be an antibody passively transferred from the donor to the recipient rabbits (*b*) antibodies could have been formed to a trace of antigen still present in the donor serum too small to be detected by the methods employed (*c*) antibodies could have been formed to trace impurity of BPG which escaped detection in the sera from the donor rabbits, (*d*) the injection of the precipitates obtained from the sera of donor rabbits along with Freund's adjuvants could have induced the formation of anti rabbit globulin in the recipient rabbits which might have cross reacted with BPG (*e*) antibodies could have been formed to an antigen like substance which can act as a substitute for the original antigen in immunization

Material isolated by alkali treatment is known to contain nonprecipitating antibodies converted to a precipitin like substance If following an injection into recipient rabbits part of this material became redissolved the presence of antibody in the sera could be envisaged This antibody being a nonprecipitating antibody converted to a precipitin like material can be distinguished from precipitating antibody by the following characteristics [8] (*a*) If varying amounts of antigen are added to nonprecipitating antibody converted to a precipitin like material a zone will be found in which antigen coexists with antibody in the ensuing supernates. The data of Table III show no such coexistence (*b*) Larger amounts of antigen are needed for the optimal reaction of nonprecipitating alkali treated antibody than for optimal reaction of precipitating antibody However the amounts of antigen for optimal sensitization of cells were

similar with sera from recipient rabbits and with anti BPG serum. Again the amount of antigen needed for optimal precipitin reaction of serum H was similar to that expected for precipitating antibody and was closer to the equivalence zone than it would have been in the case of nonprecipitating antibody converted to a precipitin like material. (*c*) Larger doses of antigen are needed for inhibition of the reaction of nonprecipitating antibody converted to a precipitin like material than for precipitating antibody. However comparable amounts of antigen were needed for inhibition of hemoagglutination with sera of the recipient rabbits and with anti BPG. Again the precipitin reaction with recipient serum was completely inhibited by 416 µg N antigen per milliliter while in the case of nonprecipitating antibody converted to a precipitin like material as much as 3 to 5 mg N would have been required.[16] These considerations indicate that the antibody found in the recipient rabbits represents ordinary precipitating antibody and is therefore not derived passively from the injected complex.

Quantitative considerations lend further support. The total amount of complex injected into rabbit H was 40 mg N given on days 0 (14 mg), 7 (11 mg) and 14 (15 mg). Assuming that all this injected precipitate became redissolved after transfer and assuming a half life of about 5 days for homologous globulin,[1] the amount of available globulin on the fourteenth day after the last injection would be [illegible] mg N. Table VI gives

TABLE VI. Amounts of Antibody in Rabbit H Expected from Passive Transfer Compared with Amounts Observed

Day After First Injection	Day After Last Injection	Antibody Expected (mg N/rabbit)	Antibody Found (mg N/120 ml serum)
29	15	2.7	3.6
36	22	1.0	5.0
43	29	0.39	3.0
50	36	0.14	2.0
77	63	0.0036	1.7

Rabbit H weighed 3.0 kg. The serum volume is assumed to be 40 ml/kg.

the amounts of antibody expected in rabbit H on the days the sera were examined for precipitating antibody, again assuming that the antibody is derived from passive transfer by complete redissolution of the injected complex, that this complex consisted solely of antibody globulin and that the redissolved globulin had a half life of 5 days. The table also gives the amounts of antibody actually recovered in the serum. It will be seen that more antibody was recovered than could be derived from passive transfer even if it is assumed that the observed antibody was present in the serum

only and none in the tissues and even if it is assumed that any antibody derived from redissolution of the transferred complex had the same half life as native rabbit gamma globulin rather than a half life decreased by alterations due to the alkali treatment precipitation and any redissolution *in vivo* If on the other hand it is assumed that the passively transferred complex is not redissolved completely but rather is retained at the injection site either partially or completely without appreciable decay even less soluble antibody would be expected in circulation This analysis indicates therefore that active antibody production must have occurred in the recipient rabbit

Inasmuch as a trace of antigen could have formed the small amounts of antibody elicited in the recipient rabbits existing methods for evaluation of antigen elimination may not have been sensitive enough For this reason care was taken that all the sera from the donor rabbits contained an excess of precipitating antibody Accordingly any trace of the main component of BPG persisting in the serum could not have exceeded the solubility of antibody antigen precipitates Following alkali treatment such antigen would have stayed in solution since the alkali treatment does not precipitate complexes of precipitating antibodies and antigen Also Weigle [9] has shown that the precipitates obtained by alkali treatment do not contain the original antigen The presence of even faint traces of the original antigen in the washed precipitate obtained by alkali treatment is therefore very unlikely

A trace impurity of BPG however might possibly persist and not be capable of forming antibodies in the donor rabbits which were injected intravenously but might be capable of forming antibodies when injected with Freund s adjuvants into the recipient rabbits Such traces of impurity would have been minimized but not excluded by the use of the washed precipitates formed after the alkali treatment rather than whole sera for injection into the recipient rabbits If such trace impurities would have formed antibodies in the recipient rabbits all of the antibodies would have been anti impurity antibody and none anti BPG In this case much larger amounts of BPG would have been needed for optimal sensitization of cells for the reaction with the recipient serum than for the reaction with anti BPG Furthermore if the impurity consisted of about 1 per cent of the total BPG (this would be beyond the minimal amount that can be detected in serum by precipitation with anti BPG produced with adjuvants) then about 100 times as much BPG would have been required for inhibition of agglutination with recipient serum as with anti BPG These considerations exclude therefore the possibility of a trace impurity of BPG carried over into the recipient rabbits

There is a possibility that the rabbit globulin in the precipitate from the donors became antigenic to the recipients and that such antibody

would have been detected by virtue of its cross reaction with BPG[1] However such anti rabbit globulin would have been neutralized in the recipient rabbits by the circulating rabbit globulin Furthermore the reaction would have been inhibited *in vitro* since the sera were diluted in buffer containing 1 per cent rabbit serum Finally a larger amount of BPG would have been needed for inhibition of the agglutination reaction of anti rabbit globulin than of anti BPG

The above considerations seem to exclude the possibility of a passively transferred antibody and of the formation of antibody to traces of the original antigen persisting in the precipitates used for immunization of the recipient rabbits Accordingly it appears tempting to make the conclusion that the precipitates contained a new material which is not the original antigen but nevertheless capable of forming a specific antibody to the original antigen The amounts of antiserum formed by this material are however much smaller than those formed by the original antigen Because of this the significance of the material cannot yet be evaluated properly While continued antibody formation seems to require persistence of an antigen like template[1,2,13] and while it is known that the original antigen is rapidly eliminated[6] the present experiments do not in themselves indicate whether the antigenic material described is a necessary intermediate in antibody formation Should it be proven eventually to be such an intermediate however serum would probably not be the ideal source for its isolation Perhaps the serum material obtained in the present experiments merely represents the release into circulation of a small fraction of a cellular material However unlike the transfer cells of Chase the present material does not in itself appear to be an antibody producing template Transfer cells yield an antibody qualitatively related to the antibody found in the donor The present material on the other hand is derived from precipitates containing nonprecipitating antibody converted to a precipitin like material but upon transfer produces precipitating antibody apparently by *de novo* immunization

These conclusions are in agreement with the previous observations of McMaster and his associates[10] who found persistence of antigen like material in the blood of rabbits for as long as 6 weeks and in the livers for as long as 8 weeks after a single injection of 0 1 Gm/kg of BPG This substance coexisted with antibody in the blood and acted like an antigen with rabbit anti BPG when assayed by anaphylactic shock in mice A difference existed however between the liver borne substance isolated on the fourteenth day after BPG injection and that isolated between the twenty first and twenty eighth days The latter while reacting anaphylactically in mice was unable to stimulate formation of anti BPG in rabbits The substance obtained from the livers on the fourteenth day after BPG injection was capable of reacting anaphylactically as well as

actively sensitizing the rabbits The material described in the present paper was obtained 13 to 15 days after a primary injection of antigen and 7 days after repeat injections and appears to resemble therefore the antigen of McMaster which was capable of reacting anaphylactically in mice as well as actively sensitizing rabbits Since the original injected antigen had disappeared by the thirteenth day as measured by precipitin analysis and radioactivity determinations and as suggested by the presence of free circulating antibody at least three antigens can now be distinguished in the process of antigen metabolism (1) the original antigen persisting until free antibody is detected in circulation (2) an antigen like material described by McMaster and in the present paper which is different from the original antigen but is still capable of eliciting formation of antibodies (3) an antigen like material described by McMaster present late in immunization and capable of reacting anaphylactically but no longer eliciting formation of antibodies These substances may very well represent a rather inhomogeneous distribution of decay products of the original antigen without permitting a clear distinction of three separate phases

However it may be questioned whether the antigen like component of the complex is a breakdown product of the original antigen as the complex isolated after injection of I^{131} labeled proteins in the experiments of Weigle [0] as well as in the present studies does not contain radioactivity The alternative explanation that it is a new material synthesized by the host and resembling the original antigen structurally but not chemically may perhaps deserve equal consideration

Summary

Serum was obtained from BPG immunized donor rabbits after disappearance of the injected antigen The precipitates obtained by alkali treatment from these sera were injected into recipient rabbits Antibody to BPG formed in the latter

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13

*Immunohistochemical Analysis of Hypersensitivity and Related Lesions**

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A number of poorly understood human diseases affecting primarily the connective tissues and blood vessels have at one time or another been suspected of a hypersensitivity origin. This group includes among others rheumatic fever, rheumatoid arthritis, polyarteritis nodosa, dermatomyositis, scleroderma, disseminated lupus erythematosis, glomerulonephritis, secondary amyloidosis, thrombotic thrombocytopenic purpura and bilateral renal cortical necrosis. While these diseases differ from one another in many respects they share a field of operation, the connective tissues, and have some histologic features in common. Perhaps the foremost histologic similarity among these diseases is the fibrinoid change seen in the connective tissues. This feature has been stressed by some who favor a hypersensitivity origin of some of these conditions on the basis of their morphologic similarity to experimental serum sickness, a generally accepted immunologic disorder.[6, 8, 11, 12] Others have likened most of these entities to the generalized Shwartzman phenomenon largely on the basis of this fibrinoid change and have even proposed a unifying name "Systemic Fibrinoid Diseases" for the group.[1] If some or all of the diseases have a pathogenesis similar to the Shwartzman phenomenon, their classification as a hypersensitivity disorder would be doubtful since most available information suggests that the Shwartzman phenomenon is not a hypersensitivity reaction in the usual immunologic or antigen-antibody sense.

The present immunohistochemical study was undertaken in the hope that a relatively simple analysis of the plasma protein composition of the characteristic lesions of several of the above human diseases plus experimental serum sickness and the generalized Shwartzman phenomenon might indicate some similarities or differences among these various entities. The application of histochemical techniques to this problem has

* Publication No. 159 of the Department of Pathology, University of Pittsburgh School of Medicine. This work was supported by U.S. Public Health Service Grant A-1195(C3).

yielded inconsistent and controversial results to date It was not expected that these results would have pathogenetic significance since a variety of mechanisms might lead to lesions with a similar plasma protein composition and similar mechanisms operating in different sites might elicit quite different reactions

Materials and Methods

The experimental plan involved the immunohistochemical study of the homologous albumin gamma globulin and fibrinogen or fibrin composition of the characteristic lesions obtained at biopsy and/or autopsy from patients with rheumatic fever rheumatoid arthritis disseminated lupus erythematosus secondary amyloidosis thrombotic thrombocytopenic purpura and renal cortical necrosis complicating premature separation of the placenta Characteristic lesions of serum sickness the Arthus reaction RNA and casein induced amyloidosis and the generalized Shwartzman reaction from rabbits were also studied for homologous albumin gamma globulin and fibrinogen or fibrin In addition the Arthus and serum sickness lesions were also studied for distribution of the specific antigen Neither the three antihuman sera nor the three antirabbit sera showed significant cross reactions with the specific antigens involved However the two antifibrinogen sera did have anti beta globulin components which could be eliminated by appropriate absorption Adjacent sections of all lesions were studied for the three homologous proteins with use of appropriate controls as previously described [13] In each case the staining of the lesion was compared with the staining of the remainder of the section in an attempt to determine concentration or lack of concentration of the specific protein in the lesion

Results and Comment

On the basis of these observations the conditions studied could be divided into those with lesions and particularly fibrinoid showing an increased concentration of gamma globulin and those with lesions and fibrinoid showing an increased concentration of fibrinogen or fibrin as follows

Diseases with lesions showing an increased concentration of gamma globulin

Serum sickness
Arthus reaction
Amyloidosis (human secondary and experimental)
Disseminated lupus erythematosus
Rheumatic fever
Rheumatoid arthritis

Diseases with lesions showing an increased concentration of fibrinogen or fibrin

- Generalized Shwartzman phenomenon
- Thrombotic thrombocytopenic purpura
- Bilateral renal cortical necrosis accompanying premature separation of placenta

In serum sickness not only was there a concentration of the host's gamma globulin in the arteritic and glomerulonephritic lesions but as the lesions developed antigen also was concentrated in them. This concentration of antigen in the lesions during the period when antigen antibody complexes are present in the circulation suggests that the complexes may be deposited in certain sites and call forth the characteristic reactions of serum sickness. However the distribution of gamma globulin in the lesion was much wider than the distribution of antigen suggesting that part of the gamma globulin was not associated with antigen and therefore perhaps not antibody. Search for albumin and fibrinogen revealed no concentration of these proteins in the arteritic lesions.

In the necrotic vessels infiltrated with polymorphonuclear leukocytes in the Arthus reaction there was a marked concentration of the offending antigen and a less well defined increase in gamma globulin concentration. No similar increase of albumin or fibrinogen in the lesions was observed. Again it seems likely that the deposition of antigen and gamma globulin results in part from the reaction of the intravascular antibody and the extravascular antigen in the vessel.

In experimental amyloidosis induced either by repeated casein injections which elicit a large antibody response or by repeated RNA injections which do not cause a detectable antibody response the amyloid deposits are morphologically identical. Likewise both kinds of amyloid contain large amounts of the host's gamma globulin and no demonstrable concentration of albumin or fibrinogen. In casein induced amyloid anticasein antibody and casein have been identified.[14] However whether this is the result of an immunologic reaction important in the pathogenesis of amyloidosis or whether it represents a nonspecific deposition of the host's gamma globulin some of which happens to be the specific antibody and is therefore capable of combining with the injected antigen remains to be seen. Since a very similar deposition of amyloid rich in gamma globulin can occur in the absence of a detectable antibody response it would appear that immunologic mechanisms are not essential to amyloid formation.

The immunohistochemical observation of a concentration of gamma globulin in amyloid is in agreement with the observed reaction of I^{131} labeled anti gamma globulin and amyloid and is consistent with the short half life of circulating gamma globulin in this disease.[12]

In human secondary amyloidosis the amyloid deposits show a great concentration of gamma globulin much as in experimental amyloidosis in the rabbit

In disseminated lupus erythematosus the vessel walls undergoing fibrinoid or hyaline change the LE bodies both free and in LE cells the thickened wire loop glomerular basement membranes and the splenic periarteriolar fibrosis all showed specific sharply delimited concentrations of gamma globulin with little or no evidence of an increase of albumin or fibrinogen These observations are consistent with the direct participation of the LE factor a gamma globulin in the pathogenesis of the lesions

The altered perivascular connective tissue of the myocardium in active rheumatic fever also showed an increase in gamma globulin content without any increase in albumin or fibrinogen There was no particular concentration in the Aschoff bodies per se demonstrable by this technique but rather a general increase in the involved perivascular connective tissue

The central fibrinoid change in the subcutaneous nodules of patients with rheumatoid arthritis likewise showed a specific concentration of gamma globulin Again how essential the gamma globulin may be in the pathogenesis of this disease connot be said The fact that a or hypogamma globulinemic children frequently develop a joint disease quite similar to if not the same as rheumatoid arthritis might suggest a secondary role for the gamma globulin

In contrast to the above diseases the lesions developing in the generalized Shwartzman phenomenon showed a marked concentration of fibrinogen or fibrin and little or no gamma globulin or albumin These lesions consist largely of intravascular fibrinoid or perhaps more correctly fibrin thrombi which may rapidly become incorporated into the vessel walls The fibrin nature of these thrombi has been suggested primarily on the basis of the fall in plasma fibrinogen levels at the time these thrombi are formed[8] and as a result of electron microscopic studies[10]

In thrombotic thrombocytopenic purpura the altered arterioles with hyaline fibrinoid walls and thrombi also showed a striking concentration of fibrinogen as previously described[3] with no increase in albumin or gamma globulin Whether this deposition of fibrin is primary or whether it is preceded by a change in the vessel wall cannot be said but it seems likely that the fibrinoid hyaline material in the vessel walls and thrombi represents fibrinogen or fibrin deposited from the circulation and is not primarily composed of altered constituents of the vessel wall

The resemblance of bilateral renal cortical necrosis associated with premature separation of the placenta to the generalized Shwartzman phenomenon has been noted previously[7] The renal arteries and arterioles

in this obstetrical complication showed fibrinoid change in their walls and hyaline thrombi both of which revealed a concentration of fibrinogen or fibrin without an increase in gamma globulin or albumin

Comments and Conclusions

While the above observations serve to divide the entities studied into two groups based upon the plasma protein composition of their lesions they do little to explain the pathogenetic mechanisms involved The concentration of gamma globulin in certain lesions has been cited as strong evidence for the operation of an antigen antibody mechanism Certainly the localized antigen antibody reactions which occur in the Arthus reaction and probably in serum sickness are associated with a localization of gamma globulin in the lesions However the present observations in experimental amyloidosis indicate that identical lesions with similar concentrations of gamma globulin can be achieved with two different procedures one of which is associated with an immunologic reaction and one of which is not A second possible explanation for the gamma globulin concentration is that primary connective tissue changes might increase the affinity of that tissue for gamma globulin There is normally a small affinity of gamma globulin for some tissue constituents and this might be magnified in such a situation In addition a third possible explanation is that a metabolic alteration leading to hypergamma globulinemia such as is frequently found in some of these diseases might predispose to the deposition of gamma globulin in normal or abnormal tissues

The morphologic dissimilarity between the various diseases showing gamma globulin concentration would also speak for different pathogenetic mechanisms in the different entities For example amyloidosis appears to be an entirely different type of reaction than the acute inflammatory lesions of serum sickness or rheumatic fever However the not infrequent coexistence of several of these entities in a single patient has suggested to some a common pathogenesis

On the other hand the lesions showing an increase in fibrinogen are morphologically quite similar regardless of the disease with which they are associated This might suggest a similar development of the vascular lesions in each disease the deposition of fibrin on the vessel wall and thrombosis However a variety of pathogenetic paths might lead to this final event For instance the initial injury might be to the vessel itself or to the circulating elements involved in coagulation or to both In the generalized Shwartzman phenomenon both these factors appear to play a role but much less is known of the pathogenesis of the human diseases

In our present state of ignorance regarding these so called connective tissue diseases observations such as those just presented can only serve to

point out differences and perhaps less surely similarities between some of the entities These observations do however, emphasize the point that we are far from being able to apply any unifying concept to this group of diseases

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DESIGNATED DISCUSSION

CHARLES A JANEWAY (Boston Massachusetts) It has been a privilege to sit in on today s program This morning s papers should serve to indicate the complexity of the immunochemical reagents (antigens and antibodies) and of the interactions between them which experimental pathologists have been using to explore the pathogenesis of a group of diseases which we believe may be due to hypersensitivity This afternoon we have had an opportunity to hear from and to see in a series of esthetically appealing pictures some of the results achieved by the leaders in this field of research All have made important contributions to our knowledge and offered provocative hypotheses from their observations

I cannot help congratulating Dr Latta and Dr Rebuck particularly for their contributions As so often happens a new tool can shed new light on an old problem With an instrument capable of optical resolution close to the dimensions of the individual antibody molecule Dr Latta has provided an exciting description of a pathologic process at the cellular level while Dr Rebuck has ingeniously made visible the actual sites of antigen antibody reaction on the surface of the red cell and verified the predictions of the immunologist These *in vitro* studies which indicate so clearly that antigen antibody reactions can alter cell surfaces and thus presumably release and perhaps activate intracellular enzymes are basic to an understanding of what occurs in the whole animal when an antigen reacts with antibody

The very interesting series of papers by Dr Seegal Dr Germuth Dr McKinnon and Dr Dixon concern the lesions which occur in and around blood vessels in anaphylaxis experimental serum sickness and a group of human diseases long suspected of being analogous in their pathogenesis The immunohistochemical technique of Coons and Kaplan has been used very fruitfully to explore the tissue localization of antigen and antibody in experimental diseases and of certain plasma proteins in pathological material from humans Dr Seegal s work with nephrotic sera seems clear cut and I am happy that she has been able to confirm Dr Kay s interpretation of his observations on the action of nephrotoxic duck serum in rabbits published in the thirties I find Dr Dixon s results on the collagen diseases extremely provocative and interesting and I should like to be able to confirm them Unfortunately we have not been able to demonstrate human gamma globulin in the lesions of human glomerulonephritis amyloidosis or lupus erythematosus

I don t know why there is this discrepancy The fluorescent antibody technique of immunohistochemistry is full of pitfalls as my colleague

Dr David Gitlin has taught me When one considers that antigens and antibodies even from single animals let alone pools derived from several animals are heterogeneous, that the proportions of each may determine solubility of the resulting complexes, that normal serum proteins are distributed throughout the connective tissues of the body and concentrate in areas of inflammation he appreciates that there are formidable problems in interpretation of what one sees Over and above these theoretical problems there are technical difficulties such as variations in intensity of the source of ultraviolet light and the number of washings applied to the tissues before staining which might well account for differences in results from one laboratory to another These can only be resolved as we compare notes on techniques and try to confirm one another s work more fully In any case it is clear that a great deal of first rate work is going on in an effort to carry us to a point where we can actually define why a circulating foreign protein produces injury to the tissues when antibody is being formed against it At present we know a lot more details but we haven t added much to the basic hypotheses put forward by von Pirquet and Schick in their classic monograph on serum sickness published in 1905

Victor A Najjar (Nashville Tennessee) I wish to discuss the mechanism of antibody antigen interaction and its implication in allergy *

In previous publications[3,5] we have shown that when an antibody reacts with a protein antigen there results a simultaneous alteration of the surface structure of both reacting molecules The indications for the change in configuration became obvious with the repeated demonstration that new antigenic sites develop on either surface of the two interacting moieties of the antibody antigen complex[1,3,5] This concept is clearly in harmony with known behavior of protein molecules

Quite a few years ago Pedersen[7] showed that protamines such as salmine and clupeine when they react with a number of proteins egg albumin and serum proteins not only produce association compounds, protamine protein complexes but also cause enough distortion to result in dissociation products with a much lower sedimentation constant than the parent protein The dissociated molecules were thought to be chemically different from the original protein molecules The reaction between protamines and the above proteins is not as avid as that between antibody and antigen since the former is readily reversible It is not surprising therefore that antibody antigen interaction should result in surface distortion We are in fact now searching for possible fragmentation of the participant molecules

Study supported by a research grant (PHS A 1968) from the Division of Research Grants National Institutes of Health U.S Public Health Service

The observation that in some antibody antigen systems there appears in the specific precipitate a preponderance of antibody molecules compared to antigen is a case in point In some instances the ratio is such as to make it hard to place comfortably so many molecules of antibody on one molecule of antigen of comparable molecular size The explanation here is that some of the antibody is virtually formed against new antigenic sites on the antibody portion of the complex Antibody molecules merely deposit on molecules of antibody that have reacted with antigen early in the sequence of events In connection with this we have been able to show again that rabbits immunized with 4 to 5 weekly subcutaneous injections each with 10 mg of homologous antibody-antigen complex prepared at equivalence (rabbit antiovalbumin ovalbumin) without the help of adjuvants produce either of two types of antibodies or both together

(1) Some sera show the type of antibodies that are directed against the antigen moiety of the complex Here the antibodies react with the free unmodified antigen though sluggishly as compared with the rate of their reactivity with the complex [3] Furthermore the reaction is inhibited in antigen excess

(2) Other sera possess the type of antibodies that are directed against the antibody portion of the complex These antibodies react only with the homologous complex and not with the antigen By contrast to the first type this reaction is not inhibited in antigen excess Figure 1 shows such a reaction Optical density measurements at 340 mμ were used to follow the rate of deposition of antibody on complex or free antigen The addition of antigen (8 μg of egg albumin) to one such antiserum resulted in no increase in turbidity for 70 minutes as shown by the broken line (C) However earlier addition of [illegible]00 μg of complex (rabbit antiovalbumin ovalbumin) to a similar reaction mixture resulted in rapid formation of turbidity (B) The rate of increase in optical density was equally rapid in the presence of 100 μg of antigen (A)

(3) Some sera contained antibodies of both types – that is antibodies that react with the complex and those that react with free antigen (ovalbumin) It is proposed that the latter are produced in response to the free antigen formed as a result of the dissociation of the antibody antigen complex used in the immunization We have been able to absorb exhaustively such sera by repeated additions of antigen When finally no further turbidity was formed the addition of complex resulted in a rapid increase in turbidity In a small series of 10 rabbits immunized with homologous complex using ovalbumin as the antigen moiety we have found antibodies against antigen alone in 3 against complex alone in 3 and against both in 4 rabbits

Our earlier work strongly suggested that antibodies to the antibody portion of the complex are formed [4] These results and those reported

Dr David Gitlin has taught me When one considers that antigens and antibodies even from single animals, let alone pools derived from several animals are heterogeneous, that the proportions of each may determine solubility of the resulting complexes that normal serum proteins are distributed throughout the connective tissues of the body and concentrate in areas of inflammation he appreciates that there are formidable problems in interpretation of what one sees Over and above these theoretical problems there are technical difficulties such as variations in intensity of the source of ultraviolet light and the number of washings applied to the tissues before staining which might well account for differences in results from one laboratory to another These can only be resolved as we compare notes on techniques and try to confirm one another's work more fully In any case it is clear that a great deal of first rate work is going on in an effort to carry us to a point where we can actually define why a circulating foreign protein produces injury to the tissues when antibody is being formed against it At present we know a lot more details but we haven't added much to the basic hypotheses put forward by von Pirquet and Schick in their classic monograph on serum sickness published in 1905

VICTOR A NAJJAR (Nashville Tennessee) I wish to discuss the mechanism of antibody-antigen interaction and its implication in allergy *

In previous publications[3 5] we have shown that when an antibody reacts with a protein antigen there results a simultaneous alteration of the surface structure of both reacting molecules The indications for the change in configuration became obvious with the repeated demonstration that new antigenic sites develop on either surface of the two interacting moieties of the antibody antigen complex[1 3 5] This concept is clearly in harmony with known behavior of protein molecules

Quite a few years ago Pedersen showed that protamines such as salmine and clupeine when they react with a number of proteins egg albumin and serum proteins not only produce association compounds protamine protein complexes but also cause enough distortion to result in dissociation products with a much lower sedimentation constant than the parent protein The dissociated molecules were thought to be chemically different from the original protein molecules The reaction between protamines and the above proteins is not as avid as that between antibody and antigen since the former is readily reversible It is not surprising therefore that antibody antigen interaction should result in surface distortion We are in fact now searching for possible fragmentation of the participant molecules

* Study supported by a research grant (PHS A 1968) from the Division of Research Grants National Institutes of Health U.S Public Health Service

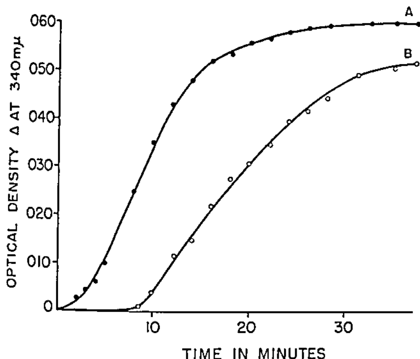


FIGURE Demonstration of inhibition of a precipitin reaction in rabbit anti serum to ovalbumin by negative rabbit antisera produced by prolonged immunization with ovalbumin The reaction mixture for curve A consisted of (*a*) 0 2 ml of rabbit antiovalbumin serum (*b*) 0 2 ml of normal rabbit serum (*c*) 0 6 ml of saline The reaction was started with 8γ of ovalbumin in 0 1 ml of saline Curve B represents a similar reaction mixture except that instead of normal serum 0 2 ml of negative nonreactive antiserum obtained from a rabbit immunized by weekly injections of ovalbumin for 67 weeks was used Both reaction cells were incubated at 26 C for 20 minutes prior to the addition of ovalbumin Optical density change was followed as in Figure 1 The increase of ovalbumin to 15γ did not alter the inhibition and represented a state of antigen excess

weeks The antisera from 3 out of 5 such rabbits showed definite inhibition of precipitin formation when incubated with precipitating sera obtained from rabbits immunized with the same antigen for only a few weeks This inhibition was quite specific to the precipitin reaction It could not be removed or obliterated by the addition of normal sera nor was there a visible precipitate formed by mixing inhibiting antisera with normal sera

Figure 2 shows the manner of inhibition observed The reaction again was followed as in Figure 1 by measuring increase in optical density upon the addition of antigen Curve A a control reaction shows the

increase in turbidity when antigen was added to the precipitating antiserum alone Curve B shows the inhibition caused by the negative nonreactive antiserum which was incubated for o minutes at 26° C with a similar aliquot of the same control reaction mixture prior to the addition of antigen In place of the negative antiserum a similar amount of normal serum was added to A which served as control and was treated in an identical manner We believe that the occurrence of such negative antisera or poorly positive antisera despite adequate immunization not only may be the result of poor reactivity of the animal but in many instances is due to the formation of antibody II to the antibody portion of the complex antibody I

If these two types of antibodies I and II are able to cross react in the absence of antigen then by the same token and based on the same premise of mutual alteration incident to interaction antibody I, to the antigen, would be sufficiently altered by antibody II that it could no longer react with antigen In other words the spatial arrangements of the reactive site of antibody I are altered beyond what is required for a complementary close fit The resulting failure of this antibody I to react with antigen breaks up the sequential events necessary for further formation of large complexes That means of course that antibody is present in these sera despite their seemingly low reactivity or their lack of it In point of fact, we were able to demonstrate the presence of antibodies after electrophoresis convection dissociation of the serum into various fractions Fractions T1 T5 and T6 showed positive precipitin tests[8] Likewise when these fractions were added to a precipitating antiserum obtained at the fourth week of immunization and a kinetic analysis made of the reaction it was noticed that during the first few minutes of the reaction the rate of precipitin formation was only slightly enhanced beyond the control (Figure 3) However the rate was more decidedly augmented over the control as the reaction proceeded This clearly indicates that the sites necessary for the deposition of the antibodies from fractions T5 and T6 particularly the former appeared only after the reaction proceeded to a definite stage, at which point a particular site was exposed It also means that T5 and T6 had some antibodies for the early stage of the complex particularly in T6 but much more antibodies to the later stages These fractions contain a preponderance of beta globulin Details of this work are reported elsewhere[2]

The findings discussed above and the concept developed therefrom can be extended to all immunological states *in vivo* or *in vitro* We have indicated that a foreign molecule an antigen acts as a modifier and so alters an antibody a normal molecule that in some instances it becomes antigenic In like manner a foreign protein a toxin an allergen etc may also interact with tissue components such as a normal tissue protein

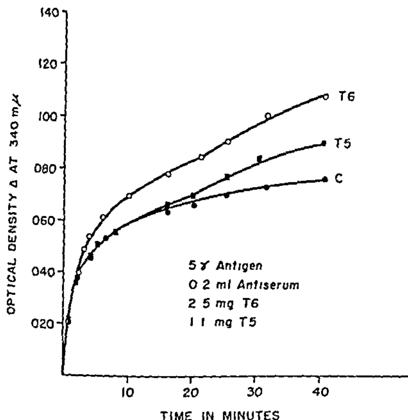


FIGURE 3 Demonstration of existence of antibodies in a negative serum The reaction mixture for control curve C consisted of 0 2 ml of antiserum from another rabbit immunized as in Figure 2 and 0 5 ml of saline T5 and T6 represented similar mixtures except that instead of saline 0 5 ml of the corresponding electrophoresis convection fractions T5 and T6 containing 1 1 and 2 5 mg of protein respectively in saline were added After incubation at 30 C for 20 minutes the reaction was started by the addition of 5γ of ovalbumin in 0 1 ml of saline Optical density increase was followed as in Figure 1

molecule Based on the same premise this interaction would result in an alteration of the normal tissue molecule If the altered configuration is not antigenic it will be of no consequence However if such alteration of the surface structure of this normal tissue molecule happens to be antigenic then antibodies are formed against it These antibodies may be highly specific and react with the tissue component only when the latter is modified by combination with the antigen In such instances the presence of antigen is obviously necessary Such was the case with the antibodies directed against the antibody portion of the complex We were unable to show an interaction of these antibodies unless antigen was present

From the clinical standpoint we picture the allergic state as belonging to this category An individual who is allergic to ragweed pollen may occasionally have antibodies to the ragweed antigen However, ordinarily the preponderance of antibodies is of the type that can be detected only by passive transfer tests We picture these to be directed in the main against an altered normal tissue protein rendered such by interaction with ragweed antigen to form an antigenic complex To produce a reaction manifested by the well known skin response, all components of the system should be present These are antibody pollen and the tissue component which should be present in the skin the test organ even though it may be also present in other tissues Sera from such allergic individuals would naturally be unreactive *in vitro* with ragweed alone since the tissue component is lacking We are now attempting to isolate a tissue component that will satisfy all *in vitro* criteria of antibody antigen reactions when mixed with ragweed pollen and antiserum

The antibodies formed to the altered tissue component would naturally have some affinity to the normal unmodified tissue molecule provided the modified site is not too alien in configuration In this case, even in the absence of the antigen, a reaction between the antibody and tissue protein may result in some measure of association between the two molecules This association would account for the prolonged residence of the antibody at the site of the skin test that is, the prepared passive transfer area It would also explain why so often one observes a local reaction in the skin following the injection of the allergic serum during passive transfer Further if this cross reaction between antibody to the modified tissue component and the normal unmodified tissue component is sufficiently strong to damage the function of the latter it would give rise to the type referred to as allergic disease This would be akin to the negative sera that possess an inhibiting antibody that is, an antibody that reacts with another antibody strongly enough to alter the latter s reactive site and therefore affects its function as an antibody (Figure) Such conditions would include allergic encephalitis nephritis etc The modifier or altering foreign substance under experimental conditions would be the adjuvant used Clinically, such an agent that modifies tissue components to render them antigenic would be a virus a toxin or some bacterial product and, in the case of serum sickness a heterologous antiserum such as horse serum

In summary then the allergic state is the result of tissue damage exerted by antibody only in the presence of the allergen which is necessary for the modification of the surface structure of a normal tissue component to produce a complementary close fit with antibody Asthma hay fever allergic skin manifestations and serum sickness fit this category All other allergic or immunologic diseases would be caused by a similar

mechanism or by strong *cross reaction* between the antibody and the normal unmodified tissue component In this sense the continuous presence of antigen is not mandatory and the disease proceeds until synthesis of antibody stops

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GENERAL DISCUSSION

CHAIRMAN NUNCESTER We have with us today a visitor Dr E F Pfeiffer from Frankfurt Germany who is doing experiments with parabiosis in rats We should like to have him tell us something about his experiments

E F PFEIFFER (Frankfurt Germany) Our experiments have been done on rats We have been interested for the past four years in the processes which may go on after the establishment of nephrotoxic serum nephritis ' in other words in whether in the blood of such a nephritic animal some factors may appear which are able to damage other kidney tissue and especially healthy kidney tissue I don t know if this will fit perfectly in this afternoon s program but from what I have heard I think it will be good to present it here

The experiments have been done in such a way that nephrotoxic nephritis has been instituted with small amounts of nephrotoxic serum from rabbits in the so called primarily nephritic rats From 3 to 5 days later these animals were unified in parabiosis with healthy partners Then there were 4 5 and 6 days before the first proteinuria appeared in the

originally healthy parabionts This can be easily understood on the basis that the capillary shunt between both animals needs 3 to 5 days to become established

Very often the changes of the blood proteins became practically the same in both animals This also can be understood on the basis of the blood shunt between the parabiotic animals, which causes the exchange of about 10 to 50 times the blood volume of the single rat from one animal to the other per day The histological lesions in the kidneys of both parabionts have been quite similar too In 9 of 13 pairs which have been evaluated in this first experiment there has been swelling of the endothelial cells in the glomeruli of the originally healthy parabionts, there also has been proliferation of the endothelial cells and there have been tubular casts

It has been possible to transfer this experimental nephritis even after an interval of 45 days between the injection and institution of the nephrotoxic nephritis in the first animal and the performance of the parabiosis[2, 3] Therefore we concluded that it is not probable that the heterologous rabbit antibodies injected into the first animal induced the nephritis in the originally healthy partners It is our belief on the contrary, that perhaps autologous tissue antibodies developed in the first animal as consequence of the cytotoxic kidney lesions or some other tissue damaging factors initiated the nephritis in the originally healthy partners

The first experiments were made in a strain of rats that were random bred Later however we worked with only inbred rats We got exactly the same results as we had before and also in the long lasting experiments with the interval of 45 days between injection of nephrotoxic serum into the first parabiont and the performance of the parabiosis Therefore some kind of homologous allergy between partners which are not identical in a genetic sense cannot be involved in our experiments

These experimental findings are not restricted to the kidney alone We instituted an experimental panophthalmitis in rats by injecting pooled anti rat eye serum from rabbits[1, 5] With this anti eye serum we produced the most beautiful lesions in all parts of the rat eye There was inflammation and infiltration of the uvea detachment of the retina and also the lens became opaque In 21 of 25 parabiotic pairs this panophthalmitis has been transferred to the originally healthy partners

The ophthalmoscopic (hyperemia of the fundus vessels appearance of precapillaries aneurysms bleedings from the capillaries perivascular sheath development of retroretinal exudate followed by detachment of the retina) and histological (hyperemia hemorrhages and infiltration of the iris the uvea the ciliary body and the retina edema of all of the tissues of the eye including the cornea retinal detachment due to retroretinal exudation) findings of the transferred ophthalmitis were similar to those

induced by injection. The t
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animals respectively.

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specific precipitates with Freund s adjuvants he immunized other rabb
He was then able to show that the resulting anticomplex sera would fo
a precipitate with the globulin of some normal rabbit sera

The component of the complex used for immunization that stimula
the formation of these antibodies is obviously the antibody portion of
antibody antigen complex Thus, the antibody obtained from the a
serum of one rabbit immunized with antigen proved to be antige
under the circumstances when injected into another rabbit in the fo
of a specific complex This simple demonstration of the antigenicity
homologous antibody is in full agreement with the results we obtain
You will recall that our rabbits were immunized in an identical mann
but without the use of adjuvants Our results also showed that an antibo
from one rabbit injected into another in the form of washed antibo
antigen complex proved to be antigenic If it had not been possible
show the antigenicity of the homologous antibody, that is from
rabbit to another the concept we proposed would have had no ba
There has not been to date a direct experimental demonstration of
antibody in one animal formed against another antibody in the sa
animal The inference is however clear that such may well be the case

Some well known relevant examples make this assumption feasi
Tissue components such as sperm testicular extracts and thyroid extra
have been shown to be antigenic in an animal when obtained from
same animal into which they were injected

MURRAY DWORETZKY (New York, New York) I should like to rais
question concerning Dr McKinnon s paper on the role of *in vi o* antig
antibody precipitation in anaphylaxis If I understood him correctly
has ascribed the anaphylactic reaction in the rabbit to thrombosis rat
than to spasm of the pulmonary arterial system I should like to
Dr McKinnon to clarify at least to the point of restating this whet
he meant that anaphylactic shock in rabbits is due to intravascular thro
bosis rather than to constriction of the pulmonary arterial system If
does believe this I should like to have him explain the observation w
known to those who work in this area that this reaction of spasm of
pulmonary arterial system can be produced in a heart lung preparat
free of all gross blood or serum

DR MCKINNON Let me answer the last part first Coca perfused
lung and then subsequently shocked the lungs and found obstruction
the flow of perfusion fluid through them but perfusion of the lungs
preparation of a heart lung preparation I think does not necessar
remove the blood I think it can be shown that with perfusion over d
decreasing amounts of serum protein can be gotten from such a lung

I think the masses I tried to show were not thrombi in the usual sense I rather think they are precipitates occurring in the animal as they do in the test tube and that they occur in the rabbit rather soon These animals were autopsied 30 minutes after they were shocked This is a concept of mechanical obstruction We can t say that spasm does not occur but mechanical obstruction is simpler for me to understand

DR. DIXON On this point when we did similar studies with an iodine labeled antigen we could find as much as 90-odd per cent of the antigen injected to shock the rabbit fixed in the lung Radiographs showed this antigen concentrated in the thrombi or emboli whatever you prefer to call them So there is a sizable amount of antigen and presumably antibody and perhaps complement precipitated in the pulmonary vessels I don t think one mechanism precludes the other Both arterial constriction and thrombosis may be operative but the large amounts of antibody needed for fatal anaphylaxis in the rabbit would suggest that immunologic thrombosis may well play an important role

KINGSLEY M STEVENS (West Point Pennsylvania) A year ago at the New York Academy of Medicine Dr John Humphrey gave some evidence that gamma globulin absorbed to the gut very strongly He could not remove this by washing with saline or buffers but he could get this out with nonisotopic gamma globulin In contrast to this serum albumin showed very little specific adsorption I should like to ask Dr Dixon if he thinks this has any relevance to the distribution of gamma globulin which he found

DR DIXON While our observations indicate an increased concentration of certain proteins in certain lesions they do not explain the mechanism of localization The process of localization has some degree of specificity since the same protein is always found in the lesions of the same disease However just how specific this process is is unknown Certainly several of the pathological entities discussed could produce similar primary or secondary changes in connective tissues which could result in fixation of a certain plasma protein The staining of each lesion for three or more different proteins should rule out any universal nonspecific fixation of one or more proteins in normal or diseased tissues

DAVID PRESSMAN (Buffalo New York) I believe it is appropriate at this time to mention that several of the observations which were reported by Dr Seegal and made with fluorescein tag methods were made previously by radioiodine methods by my collaborators Dr Eisen and Dr Korngold

For example the long half life of antibody in the kidney was measured by radioprotein methods and shown to be 20 days

In connection with the studies comparing the use of the two tags the radioactive isotope tag and a fluorescein tag it is also worth while to point out that the two labels have individual advantages The fluorescein labeled method permits much finer resolution of just where the antibody has localized

However a high concentration of antibody is required in the specific site of localization Dr Hiramoto and I have made calculations which indicate that quantities of over o to 30 gamma of antibody per gram of tissue have to be present at a site for fluorescence to be observed

With radioiodinated antibodies very much lower concentrations can be observed It was with radioiodinated techniques that we were able to show also that antibodies prepared against rat lung localized in the lung as well as in the kidney while Dr Mellors at Sloan Kettering Institute working with us was barely able to pick this up in the lung by the fluorescein technique Dr Seegal stated that there was no localization of antilung antibodies in the lung

Dr Seegal I should like to say that in my manuscript I have includ d Dr Pressman s work The short period of time for presentation prevented the mention of papers that have reported basic contributions in this field I fully agree with Dr Pressman that by his technique much smaller amounts of antigen can be recognized

Melvin H Kaplan (Cleveland Ohio) Dr Pressman s suggestion that a more sensitive method may perhaps be needed in the case of detection of nephrotoxic antibody in the lung is supported by our own observa tions Using the more sensitive indirect fluorescent antibody method we were able to find rabbit gamma globulin in the alveolar walls of the lung of rats injected with nephrotoxic serum Control rats injected with normal rabbit serum or nephrotoxic serum specifically absorbed with rat kidney homogenate were negative I believe Dr Seegal used the direct technique, which is considerably less sensitive than the indirect an globulin procedure

Dr Seegal No I used the indirect method

Dr Kaplan The procedure I am referring to involves triple layering as follows fluorescein labeled rabbit antigoat globulin is used to detect the site of interaction of goat antirabbit globulin and the rabbit nephro toxic antibody fixed in the lung

I should like to make one further point which is pertinent to Dr

Dixon's report In studies carried out in collaboration with Dr John Vaughan we noted that gamma globulin could be very frequently observed bound in the connective tissue of synovial biopsies from patients with various kinds of arthropathy including even traumatic arthritis Therefore I think Dr Dixon is quite right in advising caution about assuming that bound gamma globulin in the tissue is necessarily of immunologic significance

Finally a last remark on the use of the fluorescent antibody technique for localizing proteins in areas of fibrinoid degeneration Since fibrinoid involves alteration of the connective tissue resulting in an enhanced affinity for eosin this alteration being therefore related to a specific dye binding capacity related to charge it is not surprising that areas of fibrinoid may show marked nonspecific binding of protein Thus in our own experience fluorescein labeled antibody reacts nonspecifically with sites of fibrinoid degeneration I have no doubt that Dr Dixon has carried out the necessary controls to establish the immunologic specificity of his staining reactions however the difficulty of the technical problem involved should perhaps be mentioned

DR DIXON First of all may I agree about the possible nonspecificity of localization of proteins in fibrinoid However I would say when one uses three antisera from the same species against three different host proteins and observes consistent differences one is probably taking care of this nonspecific possibility

One other thing in answer to some of Dr Janeway's remarks Dr Germuth just reminded me that we didn't have to have fluorescein labeled antibodies to detect gamma globulin in amyloid This was done also with radioiodinated antibodies We merely reacted the radioactively labeled anti gamma globulin with the tissue sections and were able to pick up increased fixation in amyloid tissues with a Geiger counter Thus both isotope and fluorescein labeling methods would seem to be in agreement in this case

ADOLPH ROSTENBERG JR (Chicago Illinois) I should like to inquire from Dr Germuth or any of the other speakers about experiments which assert that an important fundamental difference between immediate and delayed types of allergic hypersensitivity is based on the difference in behavior between cells from animals with the two types of sensitivity when exposed to the antigen in tissue culture Cells derived from animals with only immediate type hypersensitivity are not damaged by such exposure In view of some of the reports we have heard this afternoon it is a little hard to understand this and I wonder if any of the speakers would comment on it

Dr Germuth First of all, I should like to say something about the question that was asked concerning the significance of gamma globulin localization in tissue lesions Dixon has shown that the half life of injected radioactive labeled rabbit gamma globulin is much shorter in animals with amyloid disease than in normal animals Work which has been done with the Shwartzman reaction using Thorotrast and other colloidal materials indicates very strongly that Thorotrast absorbs gamma globulin markedly There is also the problem frequently pointed out by Dr Rich that there may be a difference in the behavior and content of antibody in serum as contrasted to plasma For instance it is possible that the clotted fibrin in serum may absorb gamma globulin Thus gamma globulin localization does not necessarily imply fixation of specific antibody

Concerning the differences between the delayed type hypersensitivity and the anaphylactic type I think much work undertaken up to now clearly shows that anaphylactic type reactions do not involve a cytotoxic antibody as do delayed reactions One cannot help feeling that tuberculin type response is different from anaphylactic type response because the tissue lesions are so different There is caseation in tuberculosis and there is good evidence to believe (although it is not conclusive as yet) that this form of necrosis is due to antigen antibody interaction of the delayed type whereas in periarteritis nodosa due to hypersensitivity there are vascular lesions and connective tissue damage

Robert S Speirs (Brooklyn New York) I should like to address two questions to Dr Germuth First he showed us very beautiful plasma cells in the cornea of the actively immunized animal and I should like to know whether these plasma cells can be obtained in passively immunized rabbits

Second what type of leukocyte precedes the presence of plasma cells? I am asking because our data have clearly shown in the mouse at least that an accumulation of eosinophil granulocytes always precedes the formation of plasma cells in actively immunized animals This is not observed in passively immunized animals (*Nature,* 181 681 1958) I should like to ask if anything like this has been seen in the rabbit

Dr Germuth In the rabbit as far as we are concerned it has been terribly difficult to tell the eosinophil from the polymorph because the polymorph of the rabbit is pseudoeosinophilic Dr Gell has a stain where it appears that one can differentiate the true eosinophil from the neutrophilic polymorphonuclear leukocyte

In collaborative work initiated by Dr Ovary it appears that when a passive Arthus reaction is elicited by an antigenic material a plasma cell

response is characteristic of the older stage of the lesion On the other hand if one uses a polysaccharide material which is nonantigenic in the rabbit the plasma cell response usually does not occur

JUSTINE S GARVEY (Pasadena California) I should like to make a few remarks on the extreme toxicity which can be elicited in normal tissue immediately with soluble antigen antibody complexes

Germuth and McKinnon have shown that the soluble complexes obtained from the usual precipitin reaction give gross anaphylaxis This was found only in antigen excess and not in antibody excess We have found that the same type of reaction can be obtained in the Schultz Dale reaction [1]

More recently Dr Ishizaka has been performing skin reactions in normal guinea pigs and has shown clearly a similar type of irritating response with decomplemented serum He has ruled out the normal irritating substance that is present in all sera by carefully fractionating the serum on a starch block electrophorein before making the antigen antibody complex

It was of particular interest to him that the antigen antibody complexes in extreme antigen excess didn t give the immediate skin reaction so he began forming various complexes of an increasing antigen concentration He analyzed these complexes by electrophoretic mobility and found that it is the antigen complex of the composition AG_2 AB or more complex complexes that give the reaction

He demonstrated that these complexes definitely contain the antigen but not in a concentration of free antigen that would give the immediate reaction in a reversed anaphylaxis test doing this by starch block tech nique and using as his antigen lightly labeled S 35 bovine serum albumin

This has led to interesting speculation as to this type of complex and what it might mean in allergic types of disease

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DR GERMUTH May I add a comment about that We have seen the same effect in anaphylactic shock in guinea pigs induced by antigen antibody complexes A maximum anaphylactic response is seen with antigen anti body complexes made with two to three fold antigen excess When one

employs complexes made with twenty to forty fold antigen excess little anaphylactic activity is observed

FRANK L ADLER (New York, New York) My question is addressed to Dr Najjar

Dr Najjar the anti antibody which you postulate has been the subject of intensive research in various laboratories during the past fifty-odd years The overwhelming evidence accumulated is that such anti antibody cannot be demonstrated May I ask if you have attempted to demonstrate the postulated antigenic change in your globulin molecule by methods such as quantitative precipitin test or agar diffusion in comparing gamma globulin as it is obtained from the serum with the antibody present in your specific precipitates or recovered from your specific precipitates by solubilization by one method or another?

DR NAJJAR The evidence for an antibody to the antibody is strictly what we have shown you today Sera of rabbits immunized with rabbit antiovalbumin ovalbumin complex cause precipitation on a homologous specific complex Such antisera react only with antibody antigen complex and this reaction is not inhibited by excess antigen This as I mentioned is presumptive evidence for an antibody against the antibody portion of the complex

The other type of evidence is that sera that are seemingly negative after immunization with ovalbumin (and we have a few of those) inhibit a homologous precipitin reaction If we dissect out these antisera with electrophoresis convection we can get fractions that do not inhibit the precipitin reaction and fractions that do inhibit the precipitin reaction The first ones actually form a precipitin when antigen is added (ovalbumin) that can be measured by protein or turbidity measurement

When the fractions are mixed together and then concentrated to the original volume they again fail to produce a precipitate when antigen is added in proper amounts It is worth adding that preliminary tests with two fractions obtained from one negative serum by convection electrophoresis (T5 T6) show a positive precipitin reaction when added to one normal rabbit serum

IV

Permeability Factors

Chairman F C. MacINTOSH, Ph D (*Montreal Canada*)

Chairman's Remarks

The subject of this session is the large and remarkably heterogeneous group of substances that can be formed in the body and whose activities include the property of making the capillary wall more permeable to proteins Some of these substances certainly others more doubtfully are involved in reactions of hypersensitivity and their activation or liberation accounts in part for the local or generalized changes seen in such reactions Our speakers tonight will be dealing with two of the agents that have received most attention serotonin (5 hydroxytryptamine) and histamine These amines of relatively simple chemical structure are remarkable one might almost say notorious for the variety of cells that contain them in high concentration and also for the multiplicity of their actions Our knowledge of their metabolism and action has increased greatly in recent years largely because the time honored pharmacological methods of study have been supplemented by powerful chemical ones Our speakers have been in the forefront of these advances and we look forward to hearing from them about the progress that has been made and also about the areas of uncertainty that still exist particularly in respect of the role that each compound plays in the normal economy of the body

Not officially on our program but perhaps of equal importance are a number of other factors whose chemistry is more complicated Of especial interest are the plasma globulins discovered by Miles and his co workers which can be activated by mere storage or dilution of the plasma and which have a powerful and specific action on capillary permeability Dr Miles will be addressing us tomorrow night but I don t know whether the provocative title he has chosen – Are We Too Trigger happy "— means that he will talk about holes shot by his factors in capillaries In any case I hope he will contribute to our discussion tonight

One should also mention the polypeptides like bradykinin which can be generated from plasma protein precursors by enzymes such as trypsin plasma or kallikrein Bradykinin and its relatives seem to act more strongly on smooth muscle and pain nerve endings than on capillaries and to have no very close relationship to the permeability factors of Miles

Finally there are the substances that release serotonin or histamine or both of these together from their tissue depots and so alter capillary permeability indirectly The list of histamine releasing compounds has grown almost ludicrously long since Anrep discovered this property in curare about twenty years ago Most of these are of interest only to pharmacologists but it is undoubtedly significant that some potent histamine liberators can be extracted from tissues One of these curiously enough is serotonin, but certainly no more than a small part of the vascular effects of serotonin can be accounted for by the action of the histamine it releases Of greater significance I think are the fact that certain polpeptides release histamine and the high probability that the effect of leukotaxine preparations on capillaries is due to the histamine they set free

The study of all these factors is complicated in the most annoying way by the disparate responses observed in the usual laboratory animals One would like for example to know why dextran is a potent histamine releaser in the rat s skin but not in the dog s why the effects of polyvinylpyrrolidone are just the reverse and why neither of these has much effect on human capillaries The histamine released by these polymers comes mainly from mast cells and this is also true of the histamine released by horse serum from skin not previously sensitized in this last case it is the cat that is the responsive species At present not much can be offered in the way of explanation for these bizarre variations

A related but still more surprising phenomenon was discovered recently by my colleagues J Q Bliss and P B Stewart I should say rather that they rediscovered it for observations like those that caught their attention had been made some fifteen years earlier by Levine and State[5] and by Freeman and Schecter[3,4] These earlier workers did not pursue the matter and it was lost sight of The basic experiment is a very simple one and applies a familiar technique One takes a dog and gives it by vein a dye like Evans Blue which stains the plasma proteins so that any appreciable leakage of plasma into the tissue spaces of the skin is easily detected as a local blueing Then one injects intradermally 0 1 ml of heparinized plasma – either the dog s own plasma obtained before the blueing (autologous plasma) or plasma from another dog (nonautologous plasma) The result is always the same nonautologous plasma produces a large bright blue spot which reaches its maximum size and intensity in about 15 minutes and fades slowly over the next day or so whereas autologous plasma produces only a small blue mark along the needle track More than 300 donor recipient pairs have now been tested and there have been no exceptions to the rule as stated though there have been some quantitative variations in the response to nonautologous plasma

The interpretation of these findings presents a number of puzzles Some of the more obvious possibilities have been excluded The response to nonautologous plasma is unrelated to the presence of the dye or of the naturally occurring isoagglutinins that can be demonstrated in the plasma of some dogs and it is unnecessary for the dogs to have had a previous contact that might have sensitized the recipient to the donor s dander Dogs of the same breed react no less strongly to one another s plasma than mongrels do and the same incompatibility is found within pure lines so highly inbred that it can be calculated that homozygosity exists at 90 per cent of the loci that were heterozygous in the original purebred stock Negative results are found only when puppies in their first postnatal month are used as donors

The plasma component responsible for the permeability change moves on electrophoretic fractionation between the beta and the gamma globulins It is thus distinct from the dilution activated permeability factor of Miles and his co workers which is an alpha globulin and differs from that factor also in that its action is not antagonized by soya bean trypsin inhibitor An antihistaminic drug on the other hand does not alter the action of the dilution factor but abolishes the effect of nonautologous plasma this and other evidence strongly suggests that the increase in permeability is mainly due to the release of histamine from skin mast cells Except for this implication of histamine Bliss and Stewart have as yet been unable to unravel the sequence of events It is not easy to sup pose that every dog is supplied with ready made antibodies for the globulins of every other dog and it appears more likely that we are dealing here with a phenomenon connected in some way with immuno logical tolerance The facts of antibody synthesis and transplantation immunity must mean that the tissues acquire early in life the ability to distinguish between native and foreign protein even when the foreign protein comes from an individual of the same species The observations I have described suggest that this distinction can be clearly made within a few minutes of the introduction of the foreign protein in contrast to the delayed type of response we associate with antibody formation

Bliss and Stewart carried out parallel tests on cats rabbits and guinea pigs and in these species could detect no difference of effect between autologous and nonautologous plasma Man however in a short series of tests appeared to behave like the dog the intensity of the reaction to nonautologous plasma could not be correlated with blood group differ ences It is probable that in both man and dog the effect of nonautologous plasma on the capillaries is not limited to the skin There are some obvious implications here for blood transfusion Drs Bliss and Johns[2] have re examined the retention of transfused plasma within the dog s vascular system and have concluded that autologous plasma regularly produces a

better and more lasting expansion of the plasma than nonautologous plasma the latter often produces generalized wheahng or even circulaton collapse Dr A S V Burgen and his colleagues at the Montreal General Hospital are currently extending the investigation to human patients and here too the indications seem to be that the best plasma for transfusion would be the patient s own

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14

On the Role of Serotonin in Anaphylaxis

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The release of vasoactive substances during anaphylaxis has been widely accepted One of these substances histamine has long been implicated in the anaphylactic reaction and there is no doubt that histamine release is an important aspect of allergic manifestations in man However there are many who consider that the histamine release theory is an incomplete explanation of this phenomenon Recently 5 hydroxytryptamine (serotonin) has received a good deal of attention from investigators and the reports have ranged from enthusiastic claims that serotonin has been the solution to all remaining problems in the field to complete denials that serotonin plays any role in anaphylaxis The truth is that a few simple and direct studies in experimental animals do indicate that serotonin is released in response to antigen antibody interaction A number of other experiments on anaphylaxis utilizing various pharmacologic agents which affect serotonin are suggestive of a serotonin involvement However thus far there has been no convincing evidence linking serotonin to allergic manifestations in man

Among the earliest experiments concerning blood serotonin in anaphylaxis were those of Humphrey and Jaques[5] They showed that addition of antigen and antibody to platelet suspensions in plasma caused release of serotonin from the platelets In subsequent studies by Waalkes *et al*[11] it was shown that injection of specific antigen into sensitized rabbits resulted in the rapid appearance of both serotonin and histamine in a free form in the plasma (Figure 1) However rabbit blood platelets are unusually rich in serotonin and it was later shown[12] that the released plasma serotonin came almost entirely from platelets whereas much of the plasma histamine was derived from tissues other than platelets (Figure 2) The problem remains as to whether serotonin is released from tissues other than the platelets Release into plasma may be a poor criterion for detecting release from tissues since serotonin is much more rapidly metabolized than histamine Several times more serotonin than histamine must be administered to rabbits in order to obtain detectable amounts in plasma (Figure 3)

Attempts to show disappearance of both histamine and serotonin from tissues after anaphylaxis have not been successful [13] However variations in amine content of tissues from individual to individual are such that only large releases would have been detected In this respect serotonin

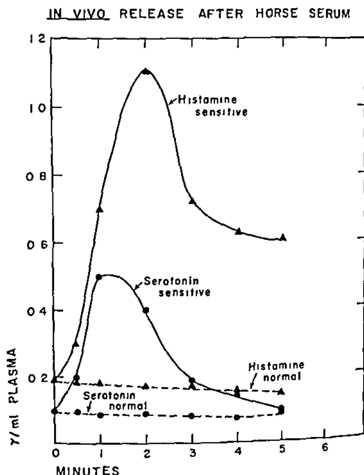


FIGURE 1 *In vivo* release after horse serum Undiluted horse serum (1 ml/kg) was injected intravenously into both sensitized and normal control rabbits anesthetized with pentobarbital and ether Plasma serotonin and histamine values were determined at the times indicated The values are the averages of 4 sensitized animals and 6 normal controls Reproduced from *J Clin Invest* 36 1115 1957

is no different from histamine There is as yet no information as to whether release of serotonin occurs during anaphylaxis in man

During anaphylaxis platelets disappear from the circulation and are apparently trapped in tissues Disappearance of platelets from the circula

tion has also been used as a test for hypersensitivity in man [10] Where do platelets go Because of their high serotonin content in certain species they can be detected in tissues Data in Figure 4 from Waalkes [7] show that in rabbits there is an increase of serotonin and histamine in the lung

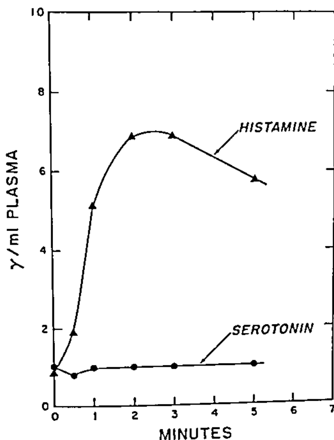


FIGURE 2 Plasma serotonin and histamine during anaphylaxis in rabbits that had received 5 mg/kg of reserpine 19 hours prior to injection of the antigen (2 ml/kg of horse serum) Values presented are the average obtained from experiments on 2 animals Reproduced from *Proc Soc Exper Biol & Med* 95 4 9 1957

accompanying the fall in circulating platelets The tissue levels return to normal as platelets return to the circulation (Table I) This tissue entrapment of platelets may have some significance It will be important to determine whether the amines in platelets produce any effects as a result of platelet disruption followed by release of amines in the organ

of entrapment As for human platelets it is known that they do disappear as a result of antigen antibody interaction [10]

In many species the lungs contain relatively large amounts of serotonin in others there is little if any (Table II) It is tempting therefore to

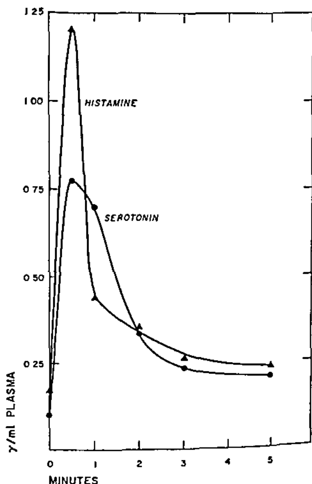


FIGURE 3 Plasma serotonin and histamine following injection of the amines. Histamine 50γ/Kg and serotonin 150γ/Kg were injected into a marginal ear vein of a normal rabbit Plasma was taken after centrifugation from blood obtained by means of a plastic catheter inserted into a carotid artery Reproduced from *J Clin Invest* 36 1115 1957

speculate about release of amines from lung depots as a factor which may influence bronchiolar tone [14] Some species like the guinea pig have little if any serotonin in the lung It is of interest that in this species anaphylaxis manifests itself entirely as a histamine reaction most prominent in

lung. Human lung contains as little serotonin as does the guinea pig. There have been some experiments, however, attempting to link serotonin to human asthmatic symptoms.[1]

Studies in animals have indicated that reactions of an anaphylactoid nature are brought about by serotonin in the skin. These studies by

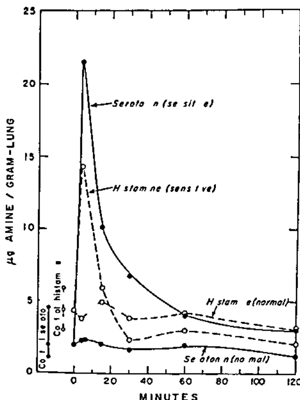


FIGURE 4. Serotonin and histamine in rabbit lung following horse serum (1 ml./Kg.)

Parratt and West[6] and by Rowley and Benditt[8] carried out on rats are extremely interesting since they do show that serotonin release is even more important than histamine in this reaction. However, before making the extrapolation to man it must be pointed out that little or no serotonin is found in the skins of species other than rats and mice (Table III). In these two rodents serotonin is found in mast cells, some of which are located in the skin. However, in man, as in most animals, mast cells are essentially devoid of serotonin.[9] Even in rodents it has not been possible to show release of significant amounts of the amines from mast cells

TABLE I Whole Blood Serotonin Histamine Platelets and Leukocytes During Anaphylaxis *

Time	Serotonin (γ/ml of whole blood)	Histamine (γ/ml of whole blood)	Platelets (no /cu mm)	Leukocytes (no /cu mm)
Control	4 5	2 3	350 000	5 000
4 min	0 8	0 6	<10 000	700
30 min	1 2	0 9	75 000	3 200
1 hour	2 1	0 9	185 000	7 800
2 hours	3 0	1 2	385 000	10 700
3 hours	3 0	1 1	330 000	13 500
4 hours	2 9	1 1	265 000	15 000

Whole blood serotonin and histamine levels and platelet and leukocyte counts were determined at specific times following the injection of diluted horse serum (1 100 in saline 1 ml /Kg) into sensitized rabbits The values given represent the average of two experiments Reproduced from *J Clin Invest* 36 1115 1957

TABLE II Serotonin and Histamine in Lung

Species	Serotonin μg /Gm	Histamine μg /Gm
Mouse	1 9	1 3
Rat	2 3	4 6
Rabbit	2 1	4 4
Guinea pig	<0 2	5–25
Dog	<0 1	25 *
Cat	<0 2	34 *
Human	<0 3	2–20

Values taken from Parratt J R and West G B *J Physiol* 137 171 1957

TABLE III Serotonin and Histamine in Skin *

Species	Serotonin μg /Gm	Histamine μg /Gm
Guinea pig	<0 02	3
Dog	<0 03	15
Rabbit	<0 04	4
Cat	0 08	24
Mouse	0 37	42
Rat	1 34	23
Man	<0 03	5

Values for these species (abdominal skin) taken from Parratt J R and West, G B., *J Physiol.,* 137 1 0 1957

including mast cell tumors, during anaphylaxis.[13] The Arthus phenomenon is a localized skin reaction in response to antigen-antibody interaction in the rabbit. It has been shown that pretreatment with reserpine, which releases serotonin and histamine, does not influence this reaction.

Some interesting reports concerning the implication of serotonin in anaphylaxis come from Fink.[4] This investigator states that anaphylaxis in the mouse can be better explained by serotonin release than by histamine release. In her investigations she makes use of the uterine smooth muscle of the sensitized mouse using the Schultz-Dale technique. Lysergic acid diethylamide and reserpine were both found to inhibit the ability of antigen to cause contraction of the sensitized uterus. Serotonin is implicated because it is a potent uterine contracting agent and the two drugs are claimed to be specific inhibitors of serotonin. Furthermore, histamine had little effect on this system. Here again the evidence is circumstantial. In our laboratory it has not been possible to demonstrate the presence of serotonin in the uterus, either in normal or sensitized animals. Moreover, although the isolated uterus is extremely sensitive to serotonin and its precursor, 5-hydroxytryptophan, they produce little if any effect on this organ *in vivo*.[3]

There is one final point of interest which, while not directly concerned with anaphylaxis, indicates that a comparison of histamine with serotonin is justified in such studies. It has been known for some time that mice which are quite insensitive to histamine become very sensitive following administration of pertussis vaccine. The mechanism of this sensitization is not known. Mice are also resistant to administered serotonin and it has now been shown[7] that pertussis vaccine increases their sensitivity to serotonin as well (Table IV). Studies by Waalkes[15] on this phenomenon have thus far failed to elucidate the mechanism for this increased sensitization. Tissues contain no more of the amines, nor do they differ in their ability to metabolize administered amines. Perhaps changes in permeability may explain the increased susceptibility to these agents.

It is apparent that many investigators are considering the possible role of serotonin in anaphylaxis. Based on animal studies in which comparisons

TABLE IV. EFFECT OF PERTUSSIS VACCINE[a] ON TOXICITIES OF SEROTONIN AND HISTAMINE IN MICE

Compound	LD_{50} (mg/kg)[b] Control	Pertussis
Serotonin	>160	20
Histamine	1750	30

[a] 2.5 × 10 bacteria.
[b] According to M. Littman.[7]

are made between histamine and serotonin there is already sufficient evidence to take serotonin release seriously as a factor in this phenomenon in many experimental animals However, there are wide differences in the distribution patterns of serotonin from species to species so that extrapolation from one species to another is dangerous Finally the drugs which are used as tools under the heading of serotonin antagonists still require much study and although some do inhibit serotonin effects under certain conditions, they can be shown to mimic serotonin in others and also to cause release of serotonin In this respect it is of interest that reserpine can cause release of histamine epinephrine, norepinephrine and 3 4 dihydroxyphenylethylamine in addition to serotonin

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15

Histamine Metabolism in the Mammalian Organism

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The subject of histamine metabolism which includes the formation of histamine and the several aspects of its fate may be divided into the following categories (1) formation ( ) binding (3) storage (4) release (5) action (6) catabolism (7) excretion In this paper recent work on some of these categories will be presented Since catabolism is perhaps the best understood and since some knowledge of it is necessary for progress in certain other categories it will be discussed first

CATABOLISM OF HISTAMINE IN MAMMALS

In all mammalian species tested there are two major histamine metabolizing enzymes One is diamine oxidase it leads to the production of imidazole 4 acetic acid and 1 ribosylimidazoleacetic acid[6] The second enzyme methylates histamine on the ring nitrogen remote from the side chain forming 1 methyl 4 (β aminoethyl) imidazole and 1 methylimidazole 4 acetic acid[1] These four metabolites will be referred to as ImAA ImAA riboside methylhistamine and MeImAA respectively

Quantitative analysis of the urine of 3 normal men[18] injected intracutaneously with 1 μg C^{14} histamine per kilogram of body weight gave

	Per Cent
Histamine	2 to 3
Methylhistamine	4 to 8
MeImAA	42 to 47
ImAA (free)	9 to 11
ImAA riboside	16 to 23

Thus in man as in most other species studied[5] MeImAA is the major end product of histamine metabolism Use of histamine metabolites in the study of histamine release will be considered in a later section

FORMATION OF HISTAMINE

Histamine seems always to be formed from *l* histidine by the action of the enzyme histidine decarboxylase There is at present no evidence

that the histamine of the tissues arises from exogenous histamine[1] by synthesis from small molecules[1] or by nonenzymatic decarboxylation of histidine

Our recent work on histidine decarboxylase has included the following studies parts of which are still in progress

(*a*) We have found that prednisone treatment produces a drop of about 80 per cent in the histidine decarboxylase of rat lung[11] If human lung behaves similarly to rat lung this finding may explain in part the beneficial effects of prednisone in asthma Histamine has been implicated as a causative agent in human asthma[25]

(*b*) The fundus of rat stomach contains extremely high histidine decarboxylase activity[16] Although histidine decarboxylase in many tissues seems to be associated with mast cells it is reported that mast cells are practically absent in the glandular portion of rat stomach[10] The role of histamine in gastric physiology is of considerable interest in view of our evidence that histamine is a gastric secretory hormone in the rat[0] and because cortisone treatment of rats increases the bound histamine of the stomach[14] The latter may have a bearing on the mechanism of formation of peptic ulcer It is known that histamine can produce ulceration in experimental animals and also that patients receiving cortisone therapy are susceptible to ulcer formation

(*c*) When rats are treated for several days with increasing doses of compound 48/80 (a powerful histamine release agent) the extractable histidine decarboxylase activity of the skin is greatly increased (6 to 10 times normal) Although mammalian adaptive enzymes are known[1] I know of no other enzyme system where a change comparable to this occurs It is not the same phenomenon as that observed by Waton[8] who found that benzene increased histidine decarboxylase activity *in vitro* We find no effect of 48/80 shortly after a single large dose or when 48 80 is incubated with rat skin histidine decarboxylase *in vitro* Also the phenomenon is not simply due to rupture of the mast cells because repeated freezing and thawing of the skin decreased histamine formation

There are two possibly important implications of these observations for research in hypersensitivity First there have been studies in which rat skin was largely depleted of histamine by repeated injections with compound 48/80 then allergic reactions were produced If depletion failed to diminish the allergic response it was held to be evidence against the participation of histamine[1] Such studies must now be re evaluated in light of the knowledge that although 48/80 depletes skin of bound histamine it also greatly increases the histidine decarboxylase activity Probably the newly developed histidine decarboxylase is in cells which have a poor binding mechanism (possibly new immature mast cells)

Otherwise it would be difficult to explain the findings that bound histamine is restored very slowly to depleted rat skin [1, 3]

Second it is known that the antigen antibody reaction as well as 48/80 treatment can release the contents of the mast cells [2, 9] If an allergic condition or skin injury of any kind through its effect on mast cells causes a rise in histidine decarboxylase activity the continuous exposure of the tissues to increased concentrations of histamine might explain some of the delayed reactions which cannot be adequately explained by the immediate action of released histamine To speculate further the time required for synthesis of new histidine decarboxylase might correspond to the time for development of delayed type hypersensitivity These hypotheses are being tested

Binding of Histamine

C^{14} histamine injected into animals is rapidly destroyed or excreted none can be detected in the tissues after a few hours (Table I) If C^{14} *l* histidine is injected C^{14} histamine can be found in some tissues for months [12] Binding of histamine *in vitro* can be demonstrated If for example rabbit platelets or rat peritoneal mast cells are incubated with C^{14} *l* histidine C^{14} histamine is formed and bound so that relatively little escapes to the supernate [13] The adrenal cortex influences the formation and binding of histamine *in vitro* In rat skin [19] and lung cortisone treatment inhibits histamine formation and binding while adrenalectomy increases it In rat stomach the reverse was found We have now been able to test the effect of the adrenals on histamine binding *in* [illegible] and have confirmed most of the *in vitro* findings

Rats were (*a*) treated with cortisone 5 mg per day each for 3 days or (*b*) adrenalectomized or (*c*) hypophysectomized (Table II) Two days after the operation (or start of cortisone treatment) they were all injected

TABLE I C^{14} Histamine in Tissues of Rats at Various Intervals after Injection of C^{14} *l* histidine

	Days				
	2	5	12	20	32
Abdominal skin	26	29	26	27	4[illegible]
Abdominal muscle	15	12	6	8	10
Stomach	73	60	25	14	14
Small intestine	17	12	6	7	4
Lung	0	—	—	—	—

Each value is the average of 6 rats Units are counts per minute per 100 mg benzenesulfonylhistamine [20] per gram of tissue

TABLE II C[14] HISTAMINE CONTENT IN RATS FOLLOWING CORTISONE ADMINISTRATION ADRENALECTOMY AND HYPOPHYSECTOMY

	Abdominal Skin	Stomach	Small Intestine	Abdominal Muscle
Control	100	100	100	100
Cortisone	23	169	46	52
Adrenalectomized	150	93	200	157
Hypophysectomized	129	87	258	130

with C[14] *l* histidine The rats were killed days later and tissues assayed for C[14] histamine The results are averages of a large number of rats and the values are relative to the controls which were arbitrarily set at 100

Thus in rats pretreated with cortisone C[14] histamine binding is decreased in all tissues except stomach where it is increased The data do not indicate that there is any hypophyseal control over histamine binding in any tissue with the possible exception of the small intestine that is not mediated by the adrenal cortex

STORAGE OF HISTAMINE

In our early experiments we injected C[14] *l* histidine into guinea pigs and determined C[14] histamine in the pooled lung intestine and kidney[1] On the basis of our findings a half life for the bound histamine in these tissues was calculated This was obviously not the best approach because each tissue may differ however there was no choice owing to the scarcity of C[14] *l* histidine

We have recently investigated the storage of C[14] histamine in individual tissues from rats Female rats were injected with 7 μc C[14] *l* histidine each Groups were killed at various intervals ranging from 2 to 3 days after injection Tissues were analyzed for C[14] histamine The average values from 6 rats per group are shown in the above table

The data show that in stomach and small intestine there is a distinct drop in histamine content with time with skin and muscle no definite drop is observed This is not surprising as stomach and intestine are metabolically active tissues with regard to protein turnover while skin and muscle are relatively inert metabolically The leveling off of the histamine content in the stomach and intestine may indicate that a portion of the histamine is contained in a metabolically inert component of these tissues Lung which has a high *in vitro* histidine decarboxylase activity contained no detectable C[14] histamine This suggests that histidine decarboxylase in rat lung may be in cells having a poor binding mechanism,

and although histamine may be formed in lung its rate of turnover is high

A very interesting question which remains to be investigated is the relative importance for allergy of new histamine compared to old histamine There are hints in the literature that new histamine is pharmacologically active in very minute quantities Goth[4] and Halpern have reported experiments which suggest that following an initial release of histamine animals restore their sensitivity to a second release of histamine in a short period of time Yet there is much evidence that the total amount of released histamine is only very slowly replaced A minute quantity of new histamine may be strategically located so that it exerts pharmacological actions far in excess of that expected from its proportion of the total histamine concentration Possibly this can be explained in some cases by the observation of Riley[11] that mast cells often start life near the blood vessels but as they mature they migrate away from the blood vessels into nearby connective tissue Thus new histamine would be that synthesized in young mast cells near blood vessels where there would be a relatively high concentration of *l* histidine for its production It is reasonable to suppose that histamine released close to blood vessels would show more pronounced pharmacological activity than histamine released in an isolated position in the connective tissue

Release of Histamine

One of the difficulties in evaluating the role of histamine in hypersensitivity is the fact that there has been no satisfactory method for measuring histamine release in living animals Small quantities of released histamine are so quickly metabolized that attempts to detect it may fail The metabolic products must be sought not histamine itself

Of the two major metabolites of histamine one imidazoleacetic acid is useless as a measure of histamine release It appears to be formed in large quantities from histidine by a second metabolic pathway not involving histamine This has been observed in rats [7] and in dogs[8] the only species tested for this reaction

The second major metabolite methylimidazoleacetic acid has the disadvantage of not giving the characteristic color reactions of imidazoles The methyl group blocks the position where these reactions occur We have taken advantage of its unreactive nature to devise a method of determination of methylimidazoleacetic acid by isotope dilution

First C^{14} histamine is incorporated into the tissues of the animal by injection of C^{14} *l* histidine A considerable portion of this histamine (when released) will be methylated and excreted as C^{14} methylimidazoleacetic acid[21] This will of course be contaminated with a huge excess of

C^{14} histidine and with its metabolic products, including imidazoleacetic acid Our procedure involves addition of carrier methylimidazoleacetic acid and preliminary purification on ion exchange resins The contaminated methylimidazoleacetic acid is then treated with benzenesulfonyl chloride The latter reacts with histidine imidazoleacetic acid and all known imidazole metabolites of histidine except methylimidazoleacetic acid The other methylated imidazoles methylhistamine and the methylhistidines react because of free amino groups in the side chain To my knowledge there would be no radioactive histidine metabolite likely to interfere which will not react with benzenesulfonyl chloride and be rendered extractable with ether The methylimidazoleacetic acid is then recovered crystallized and counted It is hoped that this technique will prove useful for evaluating histamine release in living animals

Action of Histamine

We have no direct experiments on the action of histamine We have performed experiments designed to test the mode of action of antihistamines and have found no effect on formation binding release catabolism or rate of destruction of histamine The details are presented elsewhere [17] We conclude as have most others that antihistamines act by occupying and blocking the receptors where histamine exerts its action

The manner in which histamine exerts its pharmacological action is understood even less than the other aspects of its formation and fate Until our knowledge of these phenomena is much further advanced it is not justified to exclude histamine as a causative agent in any case of hypersensitivity

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16

*The Effect of Corticosteroids upon in Vitro Blood Histamine Release**

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Blood obtained from individuals with hay fever and asthma will release histamine from the white cells into the plasma when incubated with specific antigen.[3] In these circumstances, depending upon the concentration of antigen in the blood, the amount of histamine found in the plasma may approximate ten times the control values.[10]

This reaction seems to be peculiar to the blood of so-called "atopic" individuals, for blood from patients with either immediate or serum sickness type reactions due to horse serum, antibiotics, and a parenterally administered amino acid infusion mixture has not, in our experience, shown the histamine release phenomenon when exposed to the appropriate antigen. Neither does a potent histamine liberator, compound 48/80, cause histamine release in human blood, even when concentrations causing hemolysis are used.[11]

Likewise, when histamine release occurs with the exposure of blood to specific antigen, the addition of 48/80 produces no additional release.[3]

The action of two corticosteroid compounds, prednisolone hemisuccinate and hydrocortisone acetate, upon the release of histamine produced by the exposure of blood to specific antigen *in vitro* is reported in this study.

Method

Fourteen subjects with ragweed pollinosis and 7 subjects not sensitive to ragweed were selected. By venipuncture, 100 ml. of blood was obtained from each individual. A small amount of sodium heparin was used as the anticoagulant.

* This study was supported by funds provided under Contract AF 18(600)-917 with the School of Aviation Medicine, USAF, Randolph Field, Texas, and U.S. Public Health Service Grant 1914.

The blood was divided into 5 ml aliquots and treated in the following manner

Tube I (Control) Nothing added
Tube II Ragweed antigen added
Tube III Prednisolone hemisuccinate or hydrocortisone acetate added.
Tube IV Both ragweed antigen and either prednisolone or hydrocortisone acetate added

All tubes were incubated for 40 minutes in a water bath at 37° C. To Tubes III and IV the corticosteroid was added at the start of the incubation period To Tubes II and IV ragweed antigen was added 0 minutes later All tubes were centrifuged immediately after incubation the supernatant plasma removed and plasma histamine content determined by the method of Lowry *et al*[6]

RESULTS

In Table I results of these procedures in five ragweed sensitive subjects are shown In this group the concentration of ragweed antigen was 10 000 protein nitrogen units (PNU) per 100 ml of whole blood and the concentration of prednisolone 1 0 mg per 100 ml of whole

TABLE I HISTAMINE RELEASE IN BLOOD OF RAGWEED SENSITIVE SUBJECTS (RAGWEED ANTIGEN AND PREDNISOLONE HEMISUCCINATE)

Subject	K	F	H	Pa	Hy
I Control	1	2	3	2	3
II Ragweed	14	18	16	43	47
III Prednisolone	2	2	3	2	[illegible]
IV Ragweed prednisolone	20	27	24	34	58

Figures are in micrograms of histamine per liter of plasma
Concentration of ragweed antigen 10 000 PNU/100 ml blood
Concentration of prednisolone 1 0 mg /100 ml blood

blood In all of these individuals the anticipated marked increase of histamine content was present in the samples containing ragweed antigen *when compared with the plasma histamine values of the controls* When prednisolone alone was added to blood no significant change from the control plasma histamine values was evident When prednisolone and ragweed antigen were both added however in four out of five instances a significant increase in the plasma histamine occurred when compared with the amount present with antigen alone

In four subjects (Table II) blood samples were treated as described previously except that hydrocortisone acetate was used instead of prednisolone hemisuccinate The concentration of hydrocortisone in

TABLE II HISTAMINE RELEASE IN BLOOD OF RAGWEED SENSITIVE SUBJECTS (RAGWEED ANTIGEN AND HYDROCORTISONE ACETATE)

Subject	M	Pa	R	G
I Control	3	5	2	3
II Ragweed	10	63	[illegible]	43
III Hydrocortisone	3	3	3	2
IV Ragweed hydrocortisone	13	6[illegible]	40	6[illegible]

Figures are in micrograms of histamine per liter of plasma
Concentration of ragweed antigen 10 000 PNU/100 ml blood
Concentration of hydrocortisone [illegible]0 mg /100 ml blood

whole blood was [illegible] mg per cent In blood samples from two of these subjects R and G a significant increase in release of histamine occurred when steroid was added with the antigen In a sample from a third individual M whose cells released comparatively little histamine when exposed to antigen alone the addition of hydrocortisone produced no significant additional release In the blood of the fourth individual Pa who was studied again because his blood had shown no additional release with prednisolone the presence of hydrocortisone failed to augment the histamine release produced by antigen

Four subjects not sensitive to ragweed were studied as controls using the same concentration of prednisolone In these (Table III) no significant release of histamine occurred with or without the addition of steroid to antigen

Seven ragweed sensitive subjects were studied by using smaller concentrations of both ragweed antigen and steroid than were used previously Here the concentration of added hydrocortisone was 60 μg per cent the concentration of ragweed antigen 100 PNU per cent This concentration of hydrocortisone was selected because it would not exceed concentrations obtained *in vivo* in treatment with the drug[1] The 100 PNU

TABLE III HISTAMINE RELEASE IN BLOOD OF NONALLERGIC SUBJECTS (RAGWEED ANTIGEN AND PREDNISOLONE HEMISUCCINATE)

Subject	Ne	No	L	S
I Control	2	3	2	2
II Ragweed	4	3	4	3
III Prednisolone	2	2	11	3
IV Ragweed prednisolone	5	2	2	2

Figures are in micrograms of histamine per liter of plasma
Concentration of ragweed antigen 10 000 PNU/100 ml blood
Concentration of prednisolone 1.0 mg /100 ml blood

TABLE IV HISTAMINE RELEASE IN BLOOD OF RAGWEED-SENSITIVE SUBJECTS (RAGWEED ANTIGEN AND HYDROCORTISONE ACETATE)

Subject	B	Ps	Pa	G	R	O	C
Ragweed	71	49	25	17	16	1	1
Ragweed hydrocortisone	81	60	31	27	17	2	8

Figures are in micrograms of histamine per liter of plasma
Concentration of ragweed antigen 10 000 PNU/100 ml blood
Concentration of hydrocortisone acetate 60 μg/100 ml blood

per cent concentration of ragweed antigen is said to produce a maximum release of histamine[14] These results are shown in Table IV In general the additional release of histamine is not as great with this concentration of steroid as the release achieved with greater concentrations

The exposure of whole blood to versene oxalate and other agents which remove divalent cations is known to prevent the blood histamine release reaction[6] To learn whether this inhibition also extends to the effect of steroids in augmenting histamine release blood of two ragweed sensitive subjects and three nonallergic controls was collected through the ion exchange resin column of a Fenwal Ion Exchange Blood Pack* Since this prevents coagulation no heparin was added

The results of these procedures are shown in Table V The results obtained in one of the individuals Hy whose blood also was collected with heparin in the manner previously described are shown for comparison The higher histamine control values occurring in both ragweed

TABLE V HISTAMINE RELEASE IN BLOOD COLLECTED THROUGH AN ION EXCHANGE COLUMN

Subject	Controls			Ragweed Sensitive		
	In	Wa	Bo	Co	Hy	Hy†
I Control	7	3	5	7	3	(3)
II Ragweed	8	8	6	17	4	(47)
III Prednisolone	5	3	6	6	4	(2)
IV Ragweed prednisolone	7	8	7	11	7	(58)

Figures are in micrograms of histamine per liter of plasma
Concentration of ragweed antigen 10 000 PNU/100 ml blood
Concentration of prednisolone 1 0 mg/100 ml blood
† Values in parentheses were obtained on Hy s blood not exposed to the ion exchange resin and are shown for comparison

* Provided through the kindness of Dr Bernard Bercu of the Department of Medicine Washington University School of Medicine and M R Salkeld of Ethicon Inc

sensitive and nonsensitive subjects are unexplained The lack of release in all individuals and the contrasting results when divalent cations have not been removed are evident

Discussion

The release of histamine from human blood *in vitro* is now known to depend upon several factors and undoubtedly there are many others still unknown Those which are established may be included in two groups —one concerned with donor sensitivity the other with physical and chemical factors

In general the amount of histamine released can be correlated with the subject's degree of clinical sensitivity Indeed the degree of positivity of the immediately reacting allergy skin test agrees fairly well in most instances with the degree of blood histamine release [10] This generalization is illustrated by the results of patients C and O (Table IV) who although very sensitive both clinically and by skin test to a number of other inhalants had small skin reactions to ragweed extract. This minor degree of sensitivity together with the fact that both subjects had received ragweed hyposensitization in the past probably explains the absence of histamine release in the blood samples obtained from C and O Blood from individuals treated with hyposensitization will yield a smaller histamine release [13] Adrenal cortical and probably other hormonal influences are known to have a pronounced effect on the amount of tissue histamine available for release [13]

Physical and chemical factors which have an effect on release *in vitro* include (*a*) the presence of divalent cations as illustrated by the results shown in Table V where removal of cations by exposure to an exchange resin inhibited histamine release (*b*) the temperature at which and the length of time during which the reaction is permitted to take place (*c*) the concentration of specific antigen and (*d*) the addition of inhibitors which are known enzyme poisons such as phenol and iodoacetate [8, 14]

Confirmation of these pilot observations would indicate that 17 hydroxycorticosteroids also have a significant effect upon histamine release The additional release in the presence of steroid does not always occur and the amount released varies considerably and with greater steroid concentrations may reach a value about 50 per cent greater than the amount released by antigen alone The complete lack of effect of the steroid without antigen is shown by the normal plasma histamine values in the Tube III (corticosteroid alone) controls of the ragweed sensitive individuals (Tables I and II) and in the nonallergic controls (Table III) as well Not only must the specific antigen be present but as shown in the blood samples from which divalent cations have been

removed (Table V) the release effect of the antigen must have been initiated or the steroid effect does not take place

The simplest interpretation of these results may be that the immunochemical injury to the leukocytes initiated by an antigen is increased by 17 hydroxysteroids Other leukotoxic effects which have been demonstrated by employing cortisone include the inhibition of phagocytosis and a decrease in lactic acid production[8, 7]

Hydrocortisone the chief 17 hydroxysteroid secreted by the human adrenal[11] is found *in vivo* in concentrations much smaller than those used in these studies excepting the group of experiments in which 60 µg per cent concentrations of hydrocortisone were added to the blood This concentration may exist *in vivo* when the drug is given therapeutically[2] If the counterpart of *in vitro* histamine release exists in tissues *in vivo*, it may well account for the tremendous outpouring of urinary histamine which sometimes occurs when corticosteroids are given to patients with active allergic complaints[9]

In toxic amounts histamine is regarded as a stimulator of the pituitary adrenal axis[4] However the infusion of small nontoxic amounts of histamine in human subjects will produce a temporary significant depression of plasma 17 hydroxycorticosteroid steroid levels An intriguing speculation suggests that in an *in vivo* counterpart of histamine release might have a regulatory effect upon adrenal cortical activity

Conclusion

The *in vitro* release of histamine in the blood of atopic individuals exposed to specific antigen may be increased when 17 hydroxysteroids are added

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17

Mechanism of the Anaphylactic Reaction as Studied by Means of Inhibitors

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The anaphylactic reaction can be considered as a sequence of reactions initiated by the union of antigen with cellular antibody and resulting in the release of histamine and other active substances and finally in contraction of plain muscle capillary dilatation and other physiological manifestations In the present work an attempt has been made to analyze this reaction sequence by inhibiting its course at various points[14–16]

Theoretically the anaphylactic reaction can be inhibited at three main stages The first stage the union of antigen and antibody can be inhibited by haptens low molecular substances chemically related to the antibody which combine with antibody and prevent access of the antigen In the final stages antihistamines can prevent access of released histamine on histamine receptors of plain muscle The intermediate stages following immediately upon the antigen antibody reaction can also be inhibited by both physical and chemical agents and it is this type of interference which has been most useful in elucidating the mechanism of anaphylaxis

The Failure of Antihistamines in the Anaphylactic Reaction of Plain Muscle

The ineffectiveness of antihistamines in the anaphylactic reaction of plain muscle has been extensively discussed[6, 4] and may be demonstrated for example on isolated human bronchi Figure 1 shows the effects of antihistamines on the contractions of sensitized human bronchi obtained from a patient with pollen asthma[25] Although this preparation releases histamine when treated with antigen the effects of antigen upon it are extremely resistant to antihistamines much more so than those of hista mine Several factors probably contribute to this discrepancy One is the release in anaphylaxis of slow reacting substance in addition to hista mine[1, 9] This substance is probably an acid but it has not yet been produced in a pure state Slow reacting substance is released from human and guinea pig lung in anaphylaxis but apparently acts only on

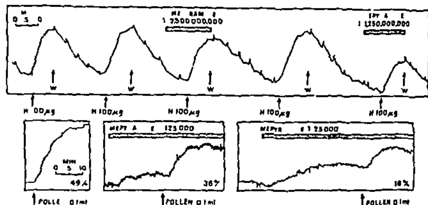


FIGURE 1 Contractions of isolated sensitized human bronchi suspended in 50 ml bath The effects of mepyramine are shown on contractions induced by histamine and by pollen Note the difference in effective concentration of mepyramine

human not on guinea pig bronchi It is therefore relevant particularly in relation to an allergic bronchoconstriction in man An interesting property of slow reacting substance is that it is formed during the anaphylactic reaction in contrast to histamine which is released from its stores but not apparently newly formed during the reaction [8]

Another factor tending to diminish the effectiveness of antihistamines is the high concentration of released histamine when it reaches the plain muscle cells This factor has been discussed before [21, 3, 4] but its significance has become clearer through recent work on the role of mast cells in the anaphylactic reaction of the guinea pig [17, 19] Mast cells undergo characteristic changes in anaphylaxis consisting of a progressive loss of mast cell granules without any morphological evidence of their fate There is no evidence of cell disruption none of the granules are seen lying outside the cell and it would seem that they become completely lysed in the course of the anaphylactic reaction In parallel with these changes the tissue becomes depleted of histamine Mast cells contain high concentrations of histamine [22] and are probably the main source of histamine in guinea pig lung and it seems possible therefore that the histamine released in anaphylaxis is derived largely from mast cell granules Mast cells are closely embedded between plain muscle cells in the guinea pig and it can be imagined that the plain muscle cells in the vicinity of mast cells are bombarded by concentrated packets of histamine in the course of the anaphylactic reaction

The effects of antihistamines upon these events must be considered in relation to the fact that they are competitive antagonists whose effects can be overcome by high concentrations of histamine such as are likely to occur in the vicinity of mast cells Only in the periphery of the source

of histamine release would the antihistamines be expected to be fully effective This may explain why these drugs produce only a partial and incomplete antagonism in anaphylaxis

While these considerations apply to a typical competitive antihistamine they need not necessarily apply to an antagonist which formed stable links with histamine receptors An antagonist which formed links with histamine receptors of the same stability as those formed by dibenamine with adrenaline receptors would probably antagonize histamine independently of concentration It would be interesting to produce a potent antagonist of this kind and test it in asthma where it might be more effective than the conventional antihistamines

Cell Structure and the Anaphylactic Reaction

A release of histamine by antigen from a sensitized tissue can only be obtained as long as the cell structure is intact[5, 13] When sensitized guinea pig lung is sliced or chopped it retains the capacity of histamine release with antigen as long as the tissue slices are large enough to contain mainly intact cells but if the chopping is carried to a point where many individual cells become damaged the anaphylactic mechanism also becomes damaged and the amount of histamine released by antigen diminishes If tissue disintegration is carried to the point where all the cells are destroyed and only the intracellular particles remain intact histamine release by antigen ceases although histamine release by octylamine and compound 48/80 is unimpaired

The failure of isolated intracellular particles to release histamine with antigen cannot be attributed to their containing a special kind of histamine which is not released in anaphylaxis On the contrary it can be proved that histamine which is released in anaphylaxis originates largely from intracellular particles it can be shown that when sensitized guinea pig lung is first exposed to antigen and then disintegrated its intracellular particles contain much less histamine than those of a control lung which has not been exposed to antigen

The histamine containing intracellular particles are concentrated in the mitochondrial fraction and are probably mainly mast cell granules If this is so the question arises why isolated mast cell granules do not react with antigen although they do so *in situ* This question cannot be answered at present in the absence of more information about the location of antibody in the mast cell it is not known where antibody is located or indeed whether mast cells contain antibody at all Assuming that they do contain antibody this could be located either on the surface or in the interior of the cell

If located on the surface the failure of isolated intracellular particles to react with antigen could be attributed to failure of the initial antigen antibody reaction on the surface which triggers off the reactions which lead to disintegration of the mast cell granules inside the cell If antibody were located inside the cell[4, 8] and perhaps on the mast cell granules themselves their failure to react with antigen could perhaps be attributed to the loss of a co factor when the cell is broken up

Histamine releasers such as octylamine act equally well on isolated particles and on intact cells This type of compound may penetrate through the cell membrane in the unionized form[11] and then act directly on the mast cell granules This view is supported by the histological observation that mast cells treated with octylamine contain a diffusely staining metachromatic mass inside[17] The picture presented by mast cells treated with octylamine is thus entirely different from that of mast cells in anaphylaxis

Methods for the Study of Inhibition of Anaphylaxis

Inhibitors of anaphylaxis can act at various sites Two types of tests are particularly useful to distinguish between these sites Tests of inhibition of histamine release distinguish between action on the effector cell and action on the histamine release mechanism and tests of desensitization distinguish between effects on the histamine release mechanism and effects on the antigen antibody reaction

It has been pointed out that sliced or chopped tissue can be used in tests for histamine release in anaphylaxis provided that the cell structure has not been destroyed In the present experiments we have used mainly the chopped guinea pig lung preparation consisting of uniform material of lung tissue in the shape of rods of about 0 5 mm cross section Many samples can be obtained from the same lung thus eliminating individual variation in sensitization and the amount of histamine released in the presence of inhibitors can be assayed biologically with considerable accuracy by the use of automatic assay apparatus (Figure 2)

Effect of Metabolic Inhibitors

Metabolic inhibitors such as iodoacetate and cyanide inhibit histamine release by antigen and potentiate it by octylamine and compound 48/80 (Figure 3) Oxygen lack acts in the same way it also inhibits histamine release in anaphylaxis and potentiates the histamine releasers This difference suggests a fundamental difference in mechanism The anaphylactic mechanism probably involves enzymatic and energy requiring

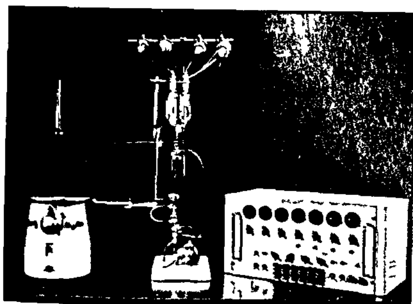


FIGURE 2 Automatic assay apparatus used for the bioassay of histamine

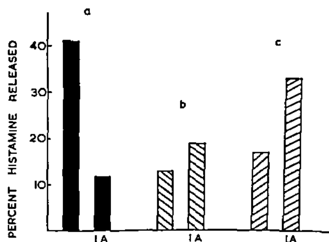


FIGURE 3 Effect of 1 mM iodoacetate on histamine release from minced sensitized guinea pig lung by (*a*) antigen (10^{-3} egg albumin) (*b*) compound 48/80 (10 [illegible]) (*c*) octylamine (2×10^{-4})

reactions whereas the histamine releasers may act by a nonenzymatic mechanism The finding that iodoacetate and other sulfhydryl reagents such as parachlormercuribenzoate block the anaphylactic reaction in fairly low concentrations (iodoacetate acts in 10^{-4} molar solution) may indicate that some reaction which involves free sulfhydryl groups is an integral part of the anaphylactic reaction Dinitrophenol on the other hand is relatively ineffective as an inhibitor of anaphylaxis and this may indicate that phosphorylated compounds are not required for the reaction However no very definite conclusions can be drawn since the known *in vitro* effects of these enzyme inhibitors cannot be simply transferred to conditions *in vivo*

A particularly interesting problem is whether the anaphylactic reaction itself is an oxidative reaction [20] The inhibitory effect of oxygen lack and cyanide on histamine release by antigen does not necessarily prove this, since oxygen may be needed simply to maintain a functioning cell rather than as a specific requirement of the anaphylactic mechanism More direct evidence has however recently been provided by experiments [12] which show that a small but definite increase in oxygen consumption occurs during the anaphylactic reaction in guinea pig lung This increase in oxygen consumption cannot be attributed to the contraction of the plain muscle of the bronchioles since histamine does not produce the same effect These findings thus provide some support for the view that an oxidative reaction is an integral part of the anaphylactic reaction

Effect of Antipyretics

Antipyretics inhibit the anaphylactic mechanism and there is some correlation between their antianaphylactic activity and their antipyretic and antirheumatic activities Salicylates which are relatively weak antipyretics are also relatively weak inhibitors of histamine release in anaphylaxis whereas amidopyrine which is a much stronger antipyretic is also much more active in inhibiting histamine release Phenylbutazone which is clinically a very powerful antipyretic and antirheumatic drug is one of the strongest inhibitors of histamine release (Figure 4)

It seems nevertheless doubtful that these antiallergic effects are specific since when these compounds are tested for other inhibitory actions—for example inhibition of oxygen consumption or of muscular contractility—they produce inhibition in about the same concentrations in which they inhibit histamine release in anaphylaxis It seems likely therefore that the latter effect is only one of the consequences of a general depression of cell function This does not of course exclude that much more specific inhibitors of anaphylaxis may in time be found

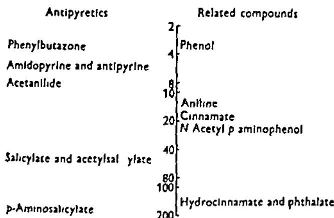


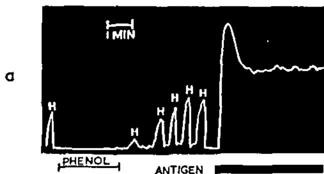
FIGURE 4 Concentration (mM) of antipyretics and related compounds for 50 per cent inhibition of histamine release in anaphylaxis

Effect of Phenol

Phenol has antipyretic activity but it is too toxic for general use It is however a very good inhibitor of histamine release in anaphylaxis and since its effects are readily reversible its mode of action has been subjected to further analysis

As pointed out before inhibitors can either inhibit the effects of active substances after they have been released or they can inhibit their release Phenol has some slight activity in inhibiting the effects of histamine on plain muscle but its main action in anaphylaxis lies in inhibiting histamine release The question then arises whether the inhibition of histamine release is due to interference with the antigen antibody reaction itself or with a subsequent event in the reaction chain which leads to histamine release

The available evidence suggests that phenol does not interfere with the antigen antibody reaction It neither inactivates antibody nor does it prevent desensitization Figure 5 shows that if sensitized guinea pig ileum is treated with antigen in the presence of phenol the anaphylactic contraction is inhibited but nevertheless if the phenol is later washed out the muscle is found to be desensitized The same phenomenon may be demonstrated for the histamine release mechanism In the presence of phenol antigen does not release histamine from sensitized lung but if the phenol is removed the tissue can be shown to have been desensitized Phenol thus has the effect of uncoupling the antigen-antibody reaction from the histamine release reaction (Figure 6)



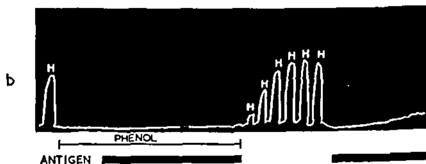


FIGURE 5 Effect of phenol on the anaphylactic reaction of the guinea pig ileum and on desensitization (*a*) Pretreatment with phenol does not produce a lasting depression of contractility and does not prevent the subsequent anaphylactic reaction (*b*) 10 mM phenol in Tyrode solution completely inhibits the anaphylactic reaction yet the tissue is desensitized after removal of the phenol H histamine 0·01 μg/ml

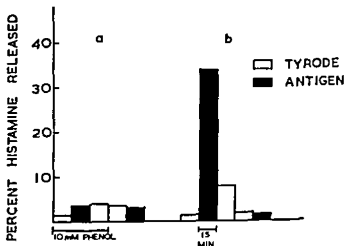


FIGURE 6 Effect of phenol on histamine release and desensitization in chopped sensitized guinea pig lung (*a*) Phenol treated sample (*b*) control Phenol reduces the histamine release by antigen from 34 to 4 per cent but it does not prevent desensitization as shown by the absence of histamine release with a second dose of antigen after removal of the phenol

Mechanism of Inhibitory Action of Phenol

A point of interest for the mechanism of the inhibitory action of phenol is that an anaphylactic reaction started in the presence of phenol fails to resume its course after removal of the phenol The point is perhaps best explained by an analogy taken from the blood clotting mechanism The last stage in the reaction of thrombin with fibrinogen consists of an aggregation process which can be inhibited by ethylene glycol If the reaction is started in the presence of ethylene glycol, fibrin forms but clotting does not occur because the final aggregation is inhibited but if the ethylene glycol is subsequently removed normal clotting takes place If phenol acted like ethylene glycol in preventing an aggregation of the antigen antibody complex it might be expected that after the removal histamine release would take place This is not the case and the following enzymatic by pass hypothesis has been proposed to resolve this difficulty [1]

It has been assumed that the combination of antigen with cellular antibody results in the activation of an enzyme system which catalyzes reactions leading to histamine release and the other manifestations of anaphylaxis The activated enzyme system is assumed to be short lived and to become rapidly inactivated An inhibitory substance such as phenol might interfere with the actions but not with inactivation of the enzyme so that in the presence of phenol histamine release would be inhibited but after removal of the phenol no free enzyme would be left to complete the reaction

Further evidence for the occurrence of an enzymatic reaction in anaphylaxis is provided by the effects of temperature and pH upon this reaction

Effects of Temperature

The temperature curve of the anaphylactic reaction is a typical enzyme curve The reaction rate as indicated by the rate of histamine release has a maximum within a narrow range around 40° C and is reduced to zero at 0° C and at 45 C (Figure 7)

Effects of Raised Temperatures

A rise in temperature of only a few degrees above normal body temperature leads to inactivation of the anaphylactic mechanism Both histamine release and the anaphylactic contraction of plain muscle are abolished If sensitized guinea pig intestine is exposed to a temperature of 45 C for 5 minutes and is then replaced into an environment of 37 C

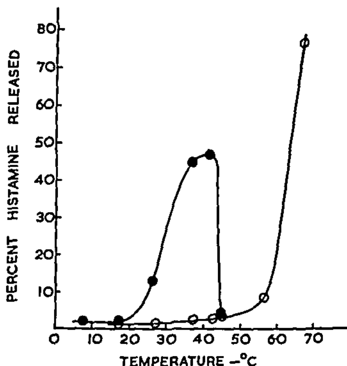


FIGURE 7 Effect of temperature on histamine release from sensitized guinea pig lung with antigen (●) and without antigen (○)

it fails to respond to antigen although it still responds to histamine (Figure 8). Similarly if sensitized guinea pig lung is heated to 45° C for 5 minutes its capacity to release histamine with antigen is destroyed.

These findings suggest that a heat labile factor is required in the anaphylactic reaction and it can be shown that this factor is not antibody but a constituent of normal tissue. The evidence for this has been provided by passive sensitization experiments *in vitro* which showed that although normal guinea pig lung could be readily sensitized passively lung which had been heated to 45° C could no longer be sensitized in this way.

The inactivation rate of the heat labile factor has been measured and the temperature coefficient of this reaction determined. The temperature coefficient is about [illegible] for one degree rise in temperature in the range 42.5° to 45° C. Such high temperature coefficients are rare but they occur in the denaturation of proteins and it was concluded that the heat labile factor is probably a protein.

The assumption of a heat labile protein fits in with the previously discussed hypothesis of the inactivation of an enzyme system as a consequence of the reaction of antigen with cellular antibody. The heat labile

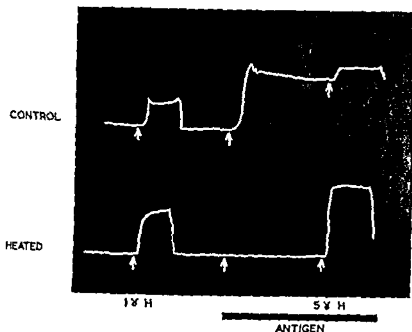


FIGURE 8 Two segments of ileum from same sensitized guinea pig in Tyrode solution at 37 C Absence of anaphylactic reaction in segment which was heated to 45 C for 5 minutes three hours before the experiment

factor may function as a proenzyme whose denaturation would block any further reaction steps

Effects of Low Temperatures

Low temperatures have complicated effects on the anaphylactic reaction they inhibit both histamine release and desensitization The histamine release mechanism is however more susceptible to cooling than the desensitization mechanism Thus at 15 C histamine release by antigen is completely inhibited but desensitization is only partially inhibited but at 0 C both are completely inhibited

Effects of Calcium and pH

The requirements of the anaphylactic reaction with regard to ions and pH are highly specific [8] Of the various cations present in Ringer s solution only calcium appears to be essential for the histamine release process in anaphylaxis In the presence of a calcium chelating agent histamine release by antigen is completely blocked The mechanism can be restored by adding calcium but not magnesium There is no evidence that magnesium or potassium is required at all both can be omitted without producing

any change even sodium does not appear to be essential since it can be replaced by sucrose without completely inhibiting the histamine release mechanism Calcium lack affects selectively the anaphylactic mechanism histamine release by octylamine is not diminished in the absence of calcium nor is the oxygen consumption of tissues appreciably reduced (Figure 9)

The anaphylactic reaction functions only in a narrow pH range and the pH activity curve resembles that of an enzyme Maximum activity

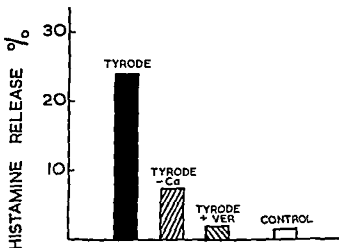


Figure 9 Effect of calcium lack on histamine release by antigen from sensitized guinea pig lung

occurs at pH 7 5 at pH 6 to 6 3 the reaction is almost completely inhibited (Figure 10)

The effects of calcium and pH are interdependent The inhibition at pH 6 can be almost completely counteracted by a tenfold increase in calcium Conversely inhibition by low calcium can be counteracted by a more alkaline pH A possible interpretation of these findings is that the anaphylactic reaction requires bound calcium and that the binding decreases with pH It is unlikely that calcium is bound to the carboxyl groups of protein for this binding is unaffected by pH in the relevant range It is possible however that calcium combines with the imidazole group of a protein since the nitrogen atom of the imidazole ring forms complexes with divalent cations which dissociate at about pH 6

Effect of Inhibitors on Mast Cell Degranulation

Inhibitors produce similar effects on histamine release and mast cell degranulation both processes are inhibited by iodoacetate phenol

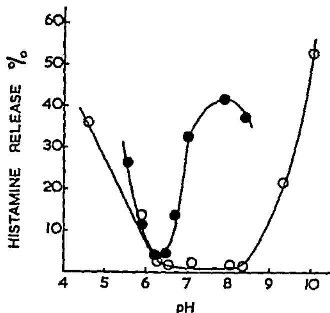


FIGURE 10 Effect of pH on histamine release from sensitized guinea pig lung with antigen (●) without antigen (○)

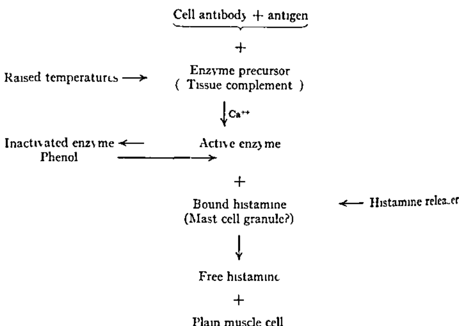
calcium lack and raised temperatures[17] Furthermore it can be shown that in the presence of an inhibitor such as phenol desensitization may take place without mast cell degranulation These findings can provide a concrete background for the enzymatic hypothesis if it is assumed that the postulated enzymatic reaction is concerned with the lysis of mast cell granules The finding that phenol blocks mast cell lysis without blocking the desensitization reaction indicates that a by pass mechanism exists as has been suggested

Relation of the Anaphylactic Reaction to the Serum Complement Reaction

The present experiments have revealed certain similarities between the anaphylactic reaction and the serum complement reaction Both reactions involve a heat labile component although the heat lability of serum complement which is inactivated at 52° C is less than that of the heat labile factor in anaphylaxis Furthermore both reactions require calcium In the serum complement reaction calcium is required for the activation and binding of the first component of complement [9] and it is possible to assume that calcium plays a somewhat similar role in anaphylaxis in being concerned with the activation of a cellular component with functions like complement

Scheme for the Anaphylactic Reaction

A possible reaction scheme which accounts for the effects of calcium and also the various other features of the anaphylactic reaction so far discussed including the effects of inhibitors is shown here



According to this scheme the antigen antibody reaction activates tissue complement in the presence of calcium The activated system has the function of an enzyme system and catalyzes reactions which lead to histamine release Since the active state is assumed to be short lived a by pass mechanism is provided which explains the blocking of histamine release but not desensitization by phenol In the absence of calcium neither histamine release nor desensitization would be expected to occur since the by pass mechanism cannot function histamine release would however be expected to occur as soon as calcium was added to the system It can indeed be shown experimentally that while there is no histamine release when the anaphylactic reaction is started in the absence of calcium histamine release does occur if at a later stage calcium ions are supplied to the system

Conclusion

The proposed scheme for the anaphylactic reaction of guinea pig plain muscle should be regarded as a working hypothesis useful mainly in

helping to devise new experiments which will no doubt have to be modified in the light of further experience Although there is strong circumstantial evidence for an enzymatic reaction in anaphylaxis direct evidence for the proposed reaction scheme is lacking no enzyme or reaction product has so far been isolated to substantiate it The present hypothesis has not so far been correlated with other suggested mechanisms of anaphylaxis such as the anaphylatoxin mechanism and the proteolytic mechanism but the various schemes are not necessarily exclusive and it is conceivable that these other mechanisms may represent further reaction steps in a complicated sequence

The main correlation so far has been with the complement reaction with which a number of similarities have been established Important differences however remain For example while calcium is required in both reactions magnesium seems to be required only for the complement reaction A close comparison between the two reactions is limited by the fact that one occurs in solution and the other is cellular and the linkage of the anaphylactic reaction to an intact cell structure makes it altogether more difficult of analysis than the complement reaction

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GENERAL DISCUSSION

G A ALIVISATOS (Chicago Illinois) An enzyme catalyzed reaction diphosphopyridine nucleotide (coenzyme I or DPN) with 4 amino carboxamido imidazole was first described by Alivisatos and Woolley was of interest to study the influence of various side chains attached carbons number 4 and/or 5 upon the ability of the imidazole to partici te in such systems Histamine [4 (or 5) (. aminoethyl) imidazole] is selected as a first example It was found that the reaction as catalyzed a soluble purified preparation of beef spleen DPN ase [6] may be scribed as follows

As shown in Figure 1 reaction 1 is essentially a nucleophilic attack of imino nitrogen of the imidazole upon carbon 1 of a ribose moiety DPN In the presence of an excess of histamine this irreversible substi tion proceeds to completion The histamine adenine dinucleotide was lated and its structure was elucidated [2] In Figure 1 the nitrogen of the idazole closest to the side chain is illustrated as participating in this iction While this is in accordance with our previous findings [1] and th the postulated mechanism of the reaction the possibility that the ier nitrogen of the imidazole is rather involved cannot be excluded present

Since histamine may be released in tissues [9] and in view of the wide tribution among animal tissues of DPN ases of the glycosidase type nilar to the one used in these experiments it could be speculated that iction 1 might be involved in the mechanism of the anaphylactic shocl cording to such a hypothesis upon release of histamine and if PN ase is present reaction 1 might occur Since the histamine adenine nucleotide cannot replace DPN in its coenzymatic activities the icken cells may then develop various degrees of damage ranging from ath by asphyxiation to milder manifestations like spasms of smooth uscular fibers and the like If such a hypothesis is true the well known fferences of sensitivity towards histamine among tissues organs or imals may be due to differences in the distribution of the enzyme PN ase It is noteworthy that the well established structural require ents for histamine like activity [7] are in harmony with the structural quirements of a nucleophilic agent for participation in reaction 1 The etabolic fate of the dinucleotide after its formation is suggested by evious findings of Karjala and of Tabor and Hayaishi [10] who isolated d identified an imidazole acetic acid riboside from the urine of animals jected with histamine Similar considerations together with the well iown pharmacological similarities of (5 hydroxy) tryptamine and of stamine led us recently to try this compound also At present however is difficult to ascertain whether reaction in Figure . catalyzed by

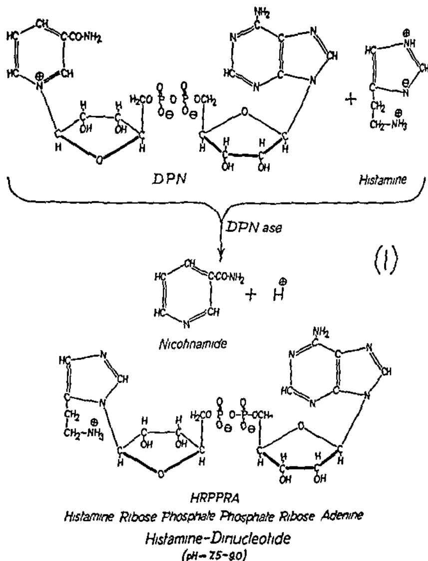
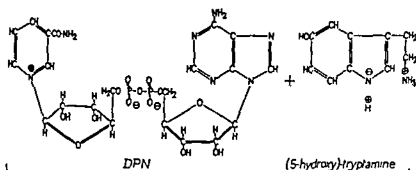
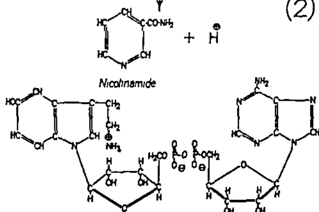

DPN
Histamine
DPNase
(I)
Nicotinamide
HRPPRA
Histamine Ribose Phosphate Phosphate Ribose Adenine
Histamine-Dinucleotide
(pH – 7.5–9.0)

FIGURE 1



Stromata of Rabbit Erythrocytes

(2)



(5-OH)-Tryptamine-Ribose Phosphate Phosphate Ribose Adenine

Serotonin-Dinucleotide

(pH—7.5 9.0)

FIGURE 2

preparations of stromata from rabbit erythrocytes [3] or beef spleen [6] might also occur Work on this subject is now in progress [4]

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BRAM ROSE (Montreal Canada) With reference to the role of histamine in anaphylaxis I think a number of observations ought to be cited

In the first place I believe everyone agrees that it is a fallacy to assume that every time an antigen combines with its antibody histamine release is an infallible result As a matter of fact it occurs only in certain types of reactions and it does not occur in all animal species

It is rather interesting that antihistamines will inhibit anaphylaxis in *some species and steroids in others* Nevertheless when anaphylaxis can be inhibited by antihistamines it is undoubtedly due to histamine release When it can be inhibited by steroids it is usually due to serotonin release

With reference to the possible significance of histamine release in the so-called allergic diseases of man I think histamine relates to the rapidly occurring manifestations such as rhinitis and asthma and in this respect it seems to be particularly significant in seasonal rhinitis due to such things as ragweed dust and the like

There is very little to support the fact that histamine release is in effect responsible for the production of asthma in man for a number of reasons None of the antihistamines are effective and it is impossible (or it has been impossible in the past at any rate) to detect significant amounts of histamine released in the blood or in the urine when such attacks are induced

On the other hand there are certain conditions in man such as hyper sensitivity to cold, where the lesion is due to the rapid release of histamine and in this circumstance it is very easy to demonstrate a very significant release of histamine into the plasma as well as an increase in the total blood histamine itself The effects can be completely inhibited by anti histamines

George L Waldbott (Detroit Michigan) My question is addressed to Dr Schayer

Sporadic abortive attempts have been made to employ histamine in treating allergic diseases especially in urticaria Some investigators have even suggested that injections with histamine might give rise to antibodies against histamine Is there anything known about the fate of histamine which has been introduced into the system and is such a theory feasible

Dr Schayer Speaking as chemist I know of no good reason why one should inject histamine to cure urticaria If you put enough histamine in you might deaden the receptors so that they are not responsive to small amounts of histamine released later over a period of time Possibly it might have something to do with adaptive growth of histamine destroying enzymes although I have never done experiments on that

Chairman MacIntosh No allergist as far as I know has yet been bold enough to try to knock out all those mast cells with powerful histamine liberators and give the patient one tremendous dose of hay fever and keep him free of it for the rest of the season

Frederick Aladjem (Los Angeles California) I should like to say a few words regarding permeability factors in human serum One simple way to demonstrate them is to isolate serum protein fractions—for instance by starch electrophoresis—and to inject these fractions intra dermally into man Within 15 minutes one can observe wheal and erythema reactions indistinguishable from those usually seen in passive transfer reactions

The serum fractions that are active migrate in the fast gamma and beta globulin region We have never observed a permeability factor in human or rabbit serums associated with the alpha globulins as Dr

Miles and his associates have reported for guinea pig serum In our experiments soybean trypsin inhibitor seemed to have little or no effect in reducing increased capillary permeability Some serums are activatable upon dilution others are not

One more point This skin test works also with autologous serum or serum fractions It is not necessary to do the test in a different subject

CHAIRMAN MACINTOSH I am interested in those comments which agree with the experience of Bliss and Stewart of our department as to the nature of the globulins involved and to some extent as to the effect of autologous plasma but in their experience the only really active samples of autologous plasma in man have been in the people who have an urticarial tendency and will give a wheal and flare with a mere injection of saline Our series however is rather small I should like to hear more later on about yours Dr Aladjem

ELI M NADEL (Bethesda Maryland) I was wondering if Dr Noah had evidence that either the hydrocortisone or the prednisolone was actually being metabolized in his *in vitro* studies and whether recovery of the steroids was attempted I bring this up because there is always a possibility that if they were not metabolized they might still have an effect merely as surface acting agents

DR NOAH I can answer that question very briefly We have made no studies of that nature

DR UDENFRIEND Just one word How specific is this Dr Noah? Do inactive steroids do this too?

DR NOAH We haven t done this with any of the steroids other than with hydrocortisone acetate and prednisolone hemisuccinate

V

Participation of Complement in Allergic Responses

Chairman IRWIN H LEPOW PH D (*Cleveland Ohio*)

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18

*Complement A Review (Including Esterase Activity)**

IRWIN H LEPOW Ph D

(Cleveland Ohio)

It is the purpose of this brief review to indicate some of the biochemical and immunological activities which have been attributed to serum complement No attempt has been made to review thoroughly the voluminous literature in this area of investigation rather it has appeared more appropriate to the purposes of this symposium to select for major emphasis the proposed enzymatic nature of complement and its participation in hypersensitivity phenomena In order to provide orientation and introduction to the nomenclature which will be employed in the papers and discussions in this section, a summary of pertinent properties of complement will also be presented

Some Properties of Serum Complement

Complement is defined by most investigators as a group of four components probably proteins present in fresh normal serum not increased by immunization and characterized by the ability to participate in antigen antibody reactions Data have been presented by several workers[1,37,39] indicating additional factors as constituents of complement † However evidence for their existence is still incomplete and complement (C) will be considered here as a four component system (C1 C C3 and C4)[8,1,84]

The total complement activity of a given serum may be estimated by its ability to lyse sheep erythrocytes sensitized with homologous rabbit antiserum It may also be estimated by quantitative measurement of the nitrogen added to specific precipitates of soluble antigen and homologous antibody by the test serum However the results obtained by each of

These investigations have been supported by Grant E-1255 from the National Institute of Allergy and Infectious Diseases of the National Institutes of Health U S Public Health Service and by a grant from Lederle Laboratories Division American Cyanamid Company Part of the work was performed as a Fellow in Pathology of the U S Public Health Service Training Grant HST 5137

† Earlier literature is cited in reference 37

these methods do not necessarily parallel each other [1] and the simpler and more functional hemolytic assay is generally employed A unit of complement in the hemolytic assay is variously defined as the smallest amount of test serum required to lyse 50 per cent or 100 per cent of a standardized suspension of optimally sensitized sheep erythrocytes

All four components of complement are required for hemolysis Procedures are available for the relatively specific inactivation or separation of each component yielding serum reagents which are hemolytically inert and which may be used therefore for the detection of the missing component Dialysis of serum against an acetate buffer of pH 5 5 and ionic strength 0 0. at 1 C for 36 to 48 hours results in a useful separation of components The first component (C 1), a euglobulin is precipitated nearly quantitatively while the second component (C 2) remains in solution In human serum, the third and fourth components (C'3 and C'4) distribute themselves essentially equally between the precipitate and supernatant In this manner two serum reagents are obtained one containing C 2 C'3 and C 4 (designated R1 a reagent for the titration of C 1) and the other C'1 C 3 and C 4 (designated R a reagent for the titration of C .) A reagent lacking C 3 (R3) may be prepared by treatment of serum with zymosan the insoluble cell wall residue of yeast Finally C 4 may be inactivated (R4) by treatment of serum with ammonia hydrazine or certain other primary amines The methods of preparation and the composition of complement components of each of the four serum reagents are summarized in Table I In addition the *relative stability to heat of each of the components is indicated*

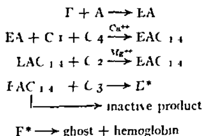
The complement reagents provide an important method for the detection and estimation of individual components of complement For example *the hemolytic activity of C'1* in an unknown serum or in a preparation of purified C 1 may be measured by adding a constant volume of various dilutions of the unknown sample to a constant optimal amount of R1

TABLE I PREPARATION AND COMPOSITION OF COMPLEMENT REAGENTS AND THE HEAT STABILITY OF COMPLEMENT COMPONENTS

Treatment of Fresh *Human Serum*	*Reagent*	Complement Component *Present*
Dialysis vs pH 5 5 acetate buffer μ = 0 02 1 36–48 hrs	Supernatant = R1 Precipitate = R2	C 2 C 3 C'4 C'1 C 3 C'4
2–3 mg zymosan per ml of serum 37 1 hr	R3	C 1 C'2 C'4
0 07–0 03 M hydrazine 37 1 hr	R4	C'1 C'2 C'3
52 30 min	—	C 1 (±) C'3 C'4
56 30 min	—	C 3 (±) C'4

and a constant volume of sensitized sheep erythrocytes and incubating at 37° C. for 30 minutes The greatest dilution of test sample which will effect 50 per cent hemolysis defines the number of units of C 1 in the sample Details of procedure and criteria for suitability of reagents are described elsewhere [1] Although it is recognized that this procedure for the titration of components of complement presents certain theoretical objections and practical difficulties reproducible and consistent data may be obtained Such component titers are not absolute values but reflect *changes* which may occur when a serum or serum fraction is treated with a given agent under controlled experimental conditions [6] In addition to their utility for measurement of components the complement reagents are valuable tools for studies on the participation of complement in a given phenomenon and the mechanism of such participation

The sequence of events in immune hemolysis has been deduced by Pillemer Ecker and co workers [34] and more recently and on a firmer kinetic basis by Mayer Levine and co workers [9] and by Leon The following reactions have been proposed by Mayer and his group



$$E + A \longrightarrow EA$$
$$EA + C1 + C4 \xrightarrow{Ca^{++}} EAC_{14}$$
$$EAC_{14} + C2 \xrightarrow{Mg^{++}} EAC_{142}$$
$$EAC_{142} + C3 \longrightarrow E^{*}$$
$$\longrightarrow \text{inactive product}$$
$$E^{*} \longrightarrow \text{ghost} + \text{hemoglobin}$$

where EA represents the sensitized sheep erythrocyte EAC_{14} and EAC_{142} represent sensitized cells which have interacted with guinea pig C 1 and C 4 and C 1 C 4 and C 2 respectively and E^{*} represents an activated or damaged erythrocyte which lyses without further participation of complement from the liquid phase These elegant studies have resulted in a clear *descriptive* picture of the reaction sequence and immune hemolysis and of the sites of participation of Ca^{++} and Mg^{++} Furthermore they have provided a basis for much more highly refined methods for the measurement of the activities of certain of the components of complement These methods should prove useful in the ultimate elucidation of the *biochemical* mechanism of immune hemolysis which in turn may serve as a model for hypersensitivity phenomena *in vivo* (see the paper by Becker p 305 in this volume)

The mechanism of the complement fixation reaction would appear at first glance to be less complex Addition of soluble antigen and homologous rabbit antibody to fresh serum results in the disappearance of

hemolytic complement activity the basis of all complement fixation tests Titrations for residual components of complement reveal, in the case of human serum complete absence of C'2 and C'4 activity, partial disappearance of C'1 and little effect of C'3 Despite serious kinetic contraindications it was generally assumed that the components of complement which disappear from solution were 'fixed' to the specific precipitate, that is complement 'fixation' was entirely an adsorption phenomenon However investigations in this laboratory have suggested an alternative mechanism, based on adsorption and activation of a proenzyme (C'1) by antigen antibody aggregates, followed by inactivation of C'2 and C'4

Enzymatic Nature of Complement

It is certainly not a fresh idea that complement may consist of one or more enzymes The enzymatic nature of complement was suggested almost seventy years ago by Buchner and various workers have associated complement with several enzymatic activities including lipases, proteases and peptidases * However this concept of complement was far from universally accepted A diametrically opposite viewpoint was held by many that complement activity was merely an expression of the colloidal state of serum Pillemer Ecker and co workers in their pioneering studies during the late 1930s and early 1940s on the nature of complement, firmly established the chemical existence of components of complement In their classical studies on the mechanism of immune hemolysis they reasoned that C'3 might be an enzyme effecting lysis *of the previously prepared erythrocyte*[34] *Data supporting* his hypothesis were not available

It was suggested several years ago on the basis of studies in this laboratory † on the mechanism of inactivation of complement by plasmin[22, 38] and by antigen antibody aggregates that the first component of human complement (C'1) exists in serum as a proenzyme[21, 26] Direct evidence was subsequently obtained by two independent approaches that C'1 is the precursor of an enzyme having esterase and complement inactivating properties

The first approach required partial purification of C'1 by a new procedure based on maintaining C'1 at high ionic strength and at pH 5 5 This fraction lost its C'1 activity when ionic strength and pH were adjusted to those of serum Simultaneously it acquired the capacity both to hydrolyze p toluenesulfonyl L-arginine methyl ester (TAMe) and to

* Earlier literature is cited in reference 34

† The original investigations summarized here are the result of a collaborative effort involving the author Dr O D Ratnoff and in earlier phases of the work, the late Dr Louis Pillemer Dr F S Rosen Miss Leona Wurz and Mr I R Levy made valuable contributions to specific parts of this investigation

inactivate the fourth component of complement C4 and to a much smaller extent the second component C'2 (Figure 1) This activity was not due to plasmin thrombin cholinesterase or acid or alkaline phosphatase

The second approach took advantage of the adsorption of C1 by aggregates of pneumococcal specific soluble substance Type III and homologous rabbit antiserum The adsorption was performed under conditions which minimized the adsorption or destruction of other components A fraction was eluted from the antigen antibody C1 complex that represented less than 0 05 per cent of the total serum nitrogen This eluate factor also hydrolyzed TAMe and inactivated C4 and to a much smaller extent C2 It did not exhibit any hemolytic complement component activities Thus the eluate factor appeared similar in its properties to those of the enzyme derived from partially purified C1 [4]

More extensive studies of substrate specificity and of kinetics have documented further the apparent identity of the enzyme derived from C1 (activated C1) and the enzyme eluted from immune aggregates [25] Both preparations hydrolyzed the same limited number of synthetic

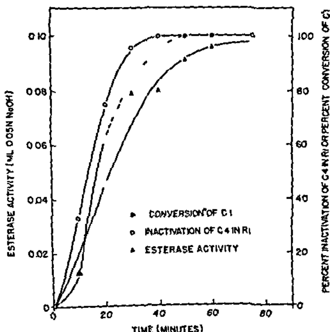


FIGURE 1 Disappearance of hemolytic C1 activity ("conversion" of C1) correlated with appearance of esterase and C'4 – inactivating properties partially purified C1 (3800 units per milliliter) incubated at 10 C at pH 7 1 ionic strength 0 15

substrates of which N-acetyl L tyrosine ethyl ester was most susceptible (Table II) Using this substrate an apparent energy of activation of 11 000 cal/mol was calculated in each case Hydrolysis was maximal at pH 7 5 to 8 and at 41° C The esterase could be inhibited by certain sulfhydryl containing reducing agents and by a heat labile factor in human serum which could not be identified with a component of complement (Table III)

TABLE II Relative Hydrolysis of Five Susceptible Substrates by Activated C 1[37]

Substrate *	Microequivalents of Acid Liberated †
N Acetyl L tyrosine ethyl ester ‡	3 6
p Toluenesulfonyl L arginine methyl ester	1 8
Benzoyl L arginine methyl ester	0 7
N Acetyl 3 5 dinitro L tyrosine ethyl ester	0 4
N Acetyl L phenylalanine ethyl ester ‡	0 0§

* All at a concentration of 0 07 M
† Incubation at pH 7 5 ionic strength 0 15 37° 15 minutes
‡ 10 per cent methyl cellosolve in reaction mixture
§ Measurable activity following more prolonged incubation

TABLE III A Comparison of the Activation of First Component to Esterase with the Hydrolysis of N Acetyl L Tyrosine Ethyl Ester by the Activated Esterase[28]

	Activation of Esterase	Esterase Activity vs N Acetyl L Tyrosine Ethyl Ester[37]
Order of reaction	Second order autocatalysis	Zero order
Energy of activation (calories per mol)	31 000	11 000
pH optimum	7 3–7 7	7 5-8 2
Effect of ionic strength (μ)	Sharply inhibited at $\mu > 0\ 15$	Gradually inhibited at $\mu > 0\ 25$
Effect of EDTA	Inhibited by 5×10^{-5}M EDTA	No effect
Serum inhibitor	Heat labile unrelated to components of complement	Heat labile unrelated to components of complement
Effect of streptokinase	Activates esterase by activation of plasminogen to plasmin	No effect

The kinetics of activation of proenzyme (C 1) to esterase have also been investigated [23] It has been found that under a wide range of physico chemical conditions a positive correlation existed between the rate of disappearance of hemolytically active partially purified C 1 and the rate of activation of an esterase hydrolyzing N acetyl L tyrosine ethyl ester Both reactions followed the kinetic equation for second order auto catalysis (Figure) and were found to have the same energy of activation (31 000 cal /mol) They occurred optimally at pH , 3 to 7 ~ and were inhibited by ionic strengths greater than 0 15 by 5×10^{-5} M ethylene diaminetetraacetic acid and by a heat labile factor in serum which appeared indistinguishable from the serum inhibitor of the active esterase

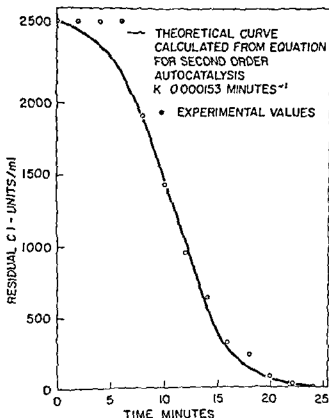


FIGURE 2 A comparison of the theoretical curve calculated from the equation for second order autocatalysis with experimental values for the rate of disappearance of hemolytically active partially purified C 1 at 20 C and at pH 7 1 ionic strength 0 15 initial concentration of C 1 of 2500 units per milliliter

The activation of C'1 to esterase resembled closely the activation of trypsinogen to trypsin (Table III)

The proenzymatic nature of C'1 has also been suggested by Becker, employing an entirely different approach[2, 3] Extending an observation of Levine[27] Becker has shown that diisopropylfluorophosphate (DFP) a known esterase inhibitor inhibits the hemolytic activity of guinea pig complement by acting on C'1 He has shown further that sensitized sheep cells containing the activities of guinea pig C'1, C'2, and C'4 are capable of hydrolyzing p toluenesulfonyl L-arginine methyl ester and that this esterase activity correlates quantitatively with the activity of C'1 on the cells Since all known esterases hydrolyzing p toluenesulfonyl L arginine methyl ester are also proteases Becker has postulated that C'1 may also be a proteolytic enzyme However aside from the possibility that C'2 and C'4 may be substrates neither proteolytic activity nor other natural substrates have been discovered thus far for activated C'1[38] Becker has also suggested that the other components of complement may be enzymes similar in their nature to C'1[3] No evidence is available to support this hypothesis

Thus evidence for the enzymatic nature of complement is restricted at this time to the proesterase role of the first component Ultimate proof will require much more highly purified preparations of C'1

Activities of Complement and Its Proposed Participation in Hypersensitivity

It has been indicated that complement is characterized by its ability to participate in antigen antibody reactions The visible or measurable manifestation of this participation is determined by the immune system under investigation Thus under appropriate conditions complement will lyse sensitized erythrocytes it will lyse or kill certain susceptible sensitized bacteria and viruses, it will promote phagocytosis of certain unsensitized bacteria and increase the rate of phagocytosis of sensitized bacteria and it will fix to specific precipitates of antigen and antibody in the complement fixation reaction * Complement or complement like factors are required for the diverse activities of the properdin system which include bactericidal and virus neutralizing properties and the lysis of certain abnormal erythrocytes[35] Complement also increases specific precipitin formation and decreases the solubility of normally soluble antigen antibody complexes[28, 32]

From the standpoint of possible mechanisms of altered reactivity the striking characteristic of complement is its ability to participate with antigen and antibody in the production of cellular injury A fundamental

* Literature is cited in reference 34

question is whether immune hemolysis and bacteriolysis *in vitro* are manifestations of a more general mechanism of cellular injury that is does complement play an active role in hypersensitivity states *in vivo*?

Excellent evidence is available which demonstrates that complement fixation occurs *in vivo* Thus Stavitsky and co workers[6] have shown a rapid and marked reduction of serum complement following reinjection of antigen into the rabbit, correlating with the presence of circulating antibody Similarly serum complement titers may be depressed during acute anaphylaxis in the guinea pig[7] and in human serum sickness[40] In a beautiful study on experimental hypersensitivity in the rabbit Schwab and co workers[41] noted a striking correlation between the time of disappearance of antigen from the circulation a reduction of serum complement, and the appearance of characteristic lesions Neither the fall in complement nor the appearance of lesions occurred if antibody formation was inhibited by administration of x radiation or nitrogen mustard

Reductions in serum complement have also been reported in several diseases which are postulated to have an allergic mechanism Thus low complement titers have been reported in disseminated lupus erythematosus acute glomerular nephritis and the nephrotic syndrome[8, 45] Curiously elevated titers were observed in anaphylactoid purpura dermatomyositis and rheumatoid arthritis[45]

These examples cited from a much larger literature leave little room for doubt that complement may participate in antigen antibody reactions *in vivo* However, they do not directly answer the critical question concerning the active or passive role of complement in mechanisms of hypersensitivity Direct approaches to this question have fallen into four categories passive anaphylaxis in the Schultz Dale phenomenon immune cytotoxicity in isolated cells or tissue culture release of histamine and serotonin from platelets and tissues by antigen antibody reactions and passive cutaneous anaphylaxis in the rat These approaches suggest an active role for complement but the need for additional data is apparent

More than fifteen years ago Kulka[17, 18] reported that the *normal* guinea pig uterine horn *passively* sensitized with antibody in the Schultz Dale apparatus would contract upon addition of homologous antigen only in the presence of fresh serum presumably complement Unfortunately this important observation has not been pursued

A rapidly increasing body of literature is accumulating documenting the requirement for fresh serum for cytotoxic effects of antibody in tissue culture* In most of these experiments the requirement for complement is determined by demonstrating that serum heated at 56 C for 30 minutes is no longer cytotoxic In at least one instance it was demonstrated further

* For example references 9 11 16 19 46

that fresh serum lost its cytotoxic activity when complement fixation was first performed with an extraneous specific precipitate[19] However more extensive studies on the identity of the cytotoxic factors in fresh serum with complement and on the mechanism of cytotoxicity are clearly indicated

Studies on the mechanism of release of histamine and serotonin in immune reactions and the role of complement are reviewed by Dr Schild in another section (for example[13 41]) Although Mongar and Schild[30 31] have presented evidence that serum complement is not involved in the liberation of histamine from guinea pig lung in anaphylaxis their proposed mechanism for these reactions[30] involving activation of a proenzyme appears strikingly similar to the proposed mechanism of complement fixation described above[19 1 5 26] The apparent lack of requirement for exogenous serum complement reported by Mongar and Schild is in agreement with the well known experiments of Dale[5] with *actively sensitized tissues in the Schultz Dale apparatus It is of interest that forty* five years ago Dale[5] wrote the proteolytic formation of the non specific poison is located by some in the circulating fluids by others in the cells participating in the anaphylactic reaction

Finally Osler and co workers[33] have implicated an active role for complement in passive cutaneous anaphylaxis in the rat These experiments which indicate a fruitful approach to the study of other hypersensitivity phenomena are summarized in Chapter 19 of this volume

It should be emphasized that different mechanisms may be operating in anaphylaxis and in the serum sickness type of hypersensitivity Thus it is entirely possible that complement may play an active role in one the other neither or both of these phenomena The current status of our knowledge has changed little since 1950 when Schwab Janeway and co workers[41] pointed out that the part complement plays in anaphylactic phenomena is not a simple one but the possibility that it may play a vital role in the pathogenesis of tissue lesions in hypersensitive states *cannot* be overlooked in our efforts to define this process in exact terms

Recapitulation and Comment

Evidence for the enzymatic nature of complement and for the participation of complement in hypersensitivity states has been reviewed briefly It has been noted that final proof of the enzymatic activity of any of the components of complement is lacking although available data are in good agreement with the proesterase nature of the first component It has also been indicated that evidence for the active role of complement in the pathogenesis of hypersensitivity phenomena is suggestive far from conclusive

It is premature to attempt to correlate these two areas of uncertainty with a unifying concept of a mechanism of hypersensitivity However it has been proposed repeatedly for more than forty years that antigen antibody reactions in blood may initiate the release of enzymatic activity which in turn may be responsible at least in part for the lesions of allergic states[4 19 14 44] It is therefore not unreasonable to speculate that if C 1 is a proesterase and if complement is actively involved in cellular injury in conditions of altered reactivity then the activation of C 1 to esterase by an antigen antibody reaction may be a step in the chain of events leading to such injury The major merit of such speculation is its susceptibility to experimental test Our current work is directed accordingly

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19

*Some Relationships between Complement, Passive Cutaneous Anaphylaxis, and Anaphylatoxin**

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Any attempt to evaluate the role of complement (C′) as a possible mediator of allergic reactions of the immediate type might well begin with an unambiguous definition of terms. Insofar as C′ is concerned, this objective is rather difficult to achieve for several reasons, the most important of which relates to the necessity of characterizing the components of C′ and their mechanism of action in operational terms.

Some of the specific limitations created by this circumstance will be considered in a later section. For the moment it may suffice to indicate that even when C′ activity estimations are confined to the lysis of sensitized erythrocytes, it cannot be assumed that the same set of experimental conditions are operative for different sources of serum C′. It is readily apparent, therefore, that the findings relating to the lysis of sheep cells treated with rabbit antibody and guinea pig serum cannot readily be extrapolated to the identification of a host factor which presumably participates in passive cutaneous anaphylaxis of the albino rat. In the light of these considerations, it might be advisable to restate some of the generally recognized properties of C′ for purposes of reference in the study of the host substance which apparently participates in allergic reactions of the immediate type. Three properties of the C′ system which are long

* This investigation from the Department of Microbiology, The Johns Hopkins School of Hygiene and Public Health and the Department of Medicine, The Johns Hopkins School of Medicine, Baltimore, was carried out under the sponsorship of the Commission on Cutaneous Diseases of the Armed Forces Epidemiological Board, and was supported in part by the Office of the Surgeon General. Support has also been received from the American Heart Association, the National Science Foundation and from a contract between the Department of Medicine, The Johns Hopkins University, and Fort Detrick, Maryland. Some of the experiments described in this manuscript were carried out with the collaboration of Miss Mary M. Hawrisiak, Miss Maria Siqueira, Dr. Otto G. Bier and Dr. Zoltan Ovary and have been published previously. We wish to thank the publishers of the *Journal of Experimental Medicine* for permission to reproduce Tables I-XI.

established, generally accepted, and pertinent to the problem at hand are

(1) The term C′ refers to a complex of at least four components present in the sera of most animals. Their activity is reportedly not increased by active immunization.

(2) C′ demonstrates a relatively low order of specificity in that it may participate in a great variety of immune phenomena.

(3) The involvement of C′ in some antigen antibody reactions may presumably proceed without any important consequences related to the immune reactants. However, in those circumstances where the antigen forms an integral component of an organized structure like a cell wall, the interaction of antibody with this antigen in the presence of C may result in destruction of the cell, be it an erythrocyte [[illegible], 74, 99] a leukocyte, a platelet [3] a treponeme [90] a Gram negative bacterium [106] a paramecium [144] and possibly a mast cell [84]. A review of the various types of lytic phenomena has recently been published by Waksman [141].

In addition to these manifold cytotoxic activities, C has also been implicated in other physiological processes ranging from blood clotting to plasma protease activation and anaphylaxis. In fact, only seven years elapsed between the descriptions of anaphylaxis by Portier and Richet [105] and the inferences drawn by Friedberger [7] and by Friedemann [37] with regard to the possible role of C in these reactions. Since that time a voluminous body of literature has accumulated on this topic. Some of the papers deal with selected and individual properties of C′ as they may relate to hypersensitivity. Other investigations bear on this question in an indirect and peripheral manner. It will be the objective of this paper to evaluate some of this evidence, not in the manner of a comprehensive review, but rather in the hope of attaining an integrated and rational perspective for further investigation.

From an historical point of view, it may be appropriate to begin with those observations bearing on the capacity of C to interact with immune aggregates, namely, C′ fixation.

In Vivo Fixation of C

The published reports considered under this heading fall into two groups differing slightly in orientation as follows

(1) The demonstration of lowered serum C levels as an index of an *in vivo* immune reaction. This finding has been used to implicate an immune reaction in the pathogenesis of certain disease processes.

( ) Initiation of *in vivo* C′ fixation as an experimental approach to studies of hypersensitivity.

Numerous investigators have relied on the diminished [illegible] lytic

potency of serum C as an index of an *in vivo* immune reaction [19, 69, 77, 119, 1, 4, 1, 9, 135, 136] Some of these experiments served a dual purpose in providing methods for manipulation of hemolytic C levels in the animal as well as in demonstrating that *in vivo* C fixation was a concomitant of a systemic immune reaction This type of experiment may be considered to have preceded the many and varied attempts to establish the immunologic nature of certain disease processes through the demonstration of a diminished lytic potency of the serum C In some instances such as in serum sickness the observed fall in C has been amply confirmed [26] Indeed this observation has been regarded as a basis for a possible mechanism of the tissue injury [80, 1, 8] Decreases in C′ levels have also been noted in a variety of other diseases with a presumed immunologic component such as enteric infections [89] arthritic processes [1, 5, 140] pneumonia [123] glomerulonephritis [24, 87, 61, 94, 112] acquired hemolytic anemia [58] rheumatic fever [16, 33, 5, 109] and other collagen type diseases [81, 144] That diminished C′ levels were not uniformly observed may be seen in the findings of Hadjopoulos and Burbank [40] as well as in some of Fischel s studies [24, 25] One of the most striking reports in this connection concerns the markedly lowered C titers in yellow fever [11] Ecker and his colleagues attempted to implicate C′ in the pathogenesis of some human diseases but observed that the fall in C′ was less than might be expected in view of the fatal consequences in some situations [17] although they attributed the loss in hemolytic activity to an *in vivo* C′ fixation process [19] It would not seem fruitful at the present stage of knowledge concerning the mechanism of C action to attempt a reconciliation of these contradictory reports There is little question but that technical considerations such as metal requirements and period of incubation are not the same for human and for guinea pig C [51] The complexities attending these efforts to estimate and correlate C activity with human disease processes were anticipated by Browning who stated in 1913 that studies of C in disease contributed little in the way of further understanding [9]

That this was not an expression of undue pessimism may be judged from the reports published since 1913 some of which have just been mentioned In all of these studies a diminution of C activity was anticipated to parallel the severity of the disease process However one of the factors which operate against this prediction emerges from observations made in several laboratories concerning the rate of C′ regeneration Following exsanguination and exchange transfusions or blockade of the reticuloendothelial system hemolytic C levels have been observed to return to normal values within 6 hours in guinea pig [10, 18, 65] and in dogs [143] In rabbits low C levels were found for about 24 hours following intravenous injection of highly concentrated meningococcus antiserum [88] This effect may perhaps be attributed to the anticomplementary action

of gamma globulin[1] A provocative observation in this regard was made by Rice and her co workers who reported that tuberculous guinea pigs on challenge with tuberculin, *experienced a fall in serum C′ levels which* was most marked 24 hours later[126] Experimental manipulation of C′ levels by systemic immune reactions have not been strikingly successful in extending the period of C′ depletion[77 100 128 1 9 133 136] Thus as shown in the studies of Bier *et al*[8] the intravenous injection of 00 μg of antibody N and homologous antigen resulted in a dramatic decrease of C′ activity which persisted for about 2 hours[100 114] However these published studies as well as unpublished experiments conducted by Dr Bauer in our laboratory indicated that after 3 or 4 successive C′-depletion procedures the rate of return and the levels of C′ were the same in the final as in the initial depletion attempt Data of this type point to an extremely rapid synthesis or release of hemolytic C′ activity If similar conditions prevail in human disease then a continued depression of C′ activity would appear unlikely Moreover the decreased C′ levels sought by investigators would seem to require the continued presence and availability of both antigen and antibody both in fairly high concentrations (cf reference 129) a qualifying condition not very *likely to be fulfilled in many* human diseases Finally, and perhaps of major importance is the consideration that the overall loss in hemolytic C′ activity may not adequately *reflect the utilization or fixation of the individual C′ components*[45 49 103 131 1] Thus as will be shown later it is possible to destroy the lytic potency of the serum completely without detectable diminution in C′3 activity Conversely it is conceivable that loss of C′1 and C′4 activities may prove to be a much more sensitive index of an immune reaction than the loss in hemolytic activity These considerations emerge from studies by Levine[84 6 4] as summarized by Mayer,[4] and point to a potentially fruitful approach in the study of those human diseases in which antigen antibody interaction may play a pathogenetic role

Demonstrations of lowered C′ activity during experimentally induced systemic anaphylaxis have recurred since the initial reports by Friedberger[34] Friedemann[37] and Sleeswijk[134] On the basis of this association between systemic anaphylactic shock and lowered serum C′ levels Friedberger and his co workers postulated *that the anaphylactic symptoms* resulted from the interaction of antibody with antigen and C′ These workers regarded C′ as the mediator of those reactions resulting in tissue injury and proposed the so called humoral theory of anaphylaxis which involved the fixation of C′ as a preliminary step to the formation of serotoxin or anaphylatoxin In fact the original reports of Friedberger which still provide a major sector of present knowledge in this area[2 31 34 35] and that of Vay[139] extend the parallelism between anaphylaxis and immune hemolysis drawn in 1907 by Gay and Southard[38]

Friedberger was not content to rest his thesis on the association between the two events but attempted to demonstrate a causal relationship between C and anaphylaxis by inhibiting the in vivo action of C with hypertonic saline [30, 34] or by preliminary depression of serum C levels [3]. Many of these studies have been summarized by Friedberger and Wassfeiler [36]

The early attempts of Friedberger to influence the anaphylactic reaction by manipulation of serum C levels have been repeated in recent years by Bier *et al* [3]. These workers used a systemic immune reaction to lower the hemolytic activity of rat serum from about 40 C'H (50 per cent hemolytic units of complement) to less than 3 per milliliter. This was achieved by intravenous injections of protein or polysaccharide antigens, followed in a matter of minutes by the homologous rabbit antisera. Under these conditions of C depletion the animals were almost completely refractory to local anaphylactic reactions as typified by passive cutaneous anaphylaxis (PCA) [70]. Some support for a causal relationship between these two events was derived from the observation that while the C depletion procedure inhibited skin responses of rats due to local antigen antibody interaction, there was no comparable diminution of the skin blueing which followed intradermal injections by histamine or snake venom [3]

The question of C' involvement in anaphylactic reactions was pursued further to obtain data which might serve to discriminate between the coincidental fall in serum C during anaphylaxis so frequently observed and mediation of the tissue injury through the participation of C or one of its components [101]

The rationale which guided these studies was based on the fact that the complex of C components are as yet discernible only through the mediation of certain reactions as these apply to immune hemolysis [74]. With respect to the more complicated allergic reactions it was considered of importance to determine whether any significant associations might be established between PCA and C in addition to the temporal coincidence of the two as described by Bier *et al* [3]

Correlations Between C Activities and PCA

Before presenting the evidence pertaining to this relationship it might be advisable to stress the limitations of interpretations which may be derived from experimental data. The most important perhaps has already been mentioned and refers to the operational criteria required for C identification. This difficulty presents a rather stubborn obstacle to an unequivocal identification of C as a mediator of anaphylaxis for the following reasons. As will be noted below, attempts have been made to

augment the intensity of PCA reactions by injections of such reagents as hemolytically active serum. This serum is assayed for its hemolytic properties only, and when its administration is followed by a heightened cutaneous response there can be no facile assumption that the hemolytic activity of the serum is the sole determinant of this intensified reaction. The increased cutaneous response is but the final event in an as yet unnumbered series of reactions which may be mediated by serum components other than C′. In this light, then, the data which will now be presented may be conceived as repeated and varied attempts to obtain further evidence regarding the presumed association of C′ activity with PCA. This evidence may be discussed under the following five headings, each of which concerns a comparison of *in vitro* C′ properties with immediate allergic reactivity of the passively sensitized skin.

1 Serum C′ Levels and PCA

As indicated in earlier studies[3, 100] the diminution of C′ levels in rats to 5 or 10 per cent of normal values is accompanied by suppression of PCA reactivity. In an effort to demonstrate that this relationship applies to intermediate levels of C′ as well, albino rats were subjected to parenteral immune reactions with graded amounts of antibody. The data in Table I indicate that for any single level of antigen and antibody the PCA response may be related to the level of hemolytic activity in the serum.

The *in vivo* C′ depletion procedure proved useful in that the serum

TABLE I COMPARATIVE STUDIES OF C′ LEVELS AND PCA REACTIONS IN C′ DEFICIENT RATS

µg Ra anti Ea N* µg Ea N Used for Decomplementation	Average $C'H_{50}$ per ml	µg SIII Used for Challenge	Rabbit Anti SIII N Injected Intradermally µg per skin site: 10.5	3.5	1.2	0.4
			Average Response mm			
100 40	22	12	6 (4)	3 (4)	0 (4)	0 (4)
200 80	9	12	2.8 (16)	1.6 (16)	0.3 (16)	0.2 (16)
100 40	22	24	13.5 (4)	9.2 (4)	4.8 (4)	0 (4)
200 80	9	24	4 (26)	3.4 (14)	1.5 (76)	0.6 (14)
400 200	4	24	0 (3)	0 (3)	0 (3)	0 (3)
100 40	22	49	14.5 (4)	9.5 (4)	3.2 (4)	0 (4)
200 80	9	49	7.7 (8)	9.5 (4)	1.6 (4)	0 (4)
200 80	9	98	16.8 (4)	10.5 (4)†	7.5 (4)	
200 80	9	196	14.8 (4)	11.8 (4)†	9.5 (4)	
1000 100	3	211	0 (8)	0 (8)†	0 (8)	

* The rabbit antiserum in the amounts indicated was injected intravenously and followed immediately by an intraperitoneal injection of the antigen.

† Actually used 2.6 µg anti SIII N at these sites.

C levels could be manipulated to suit the experimental design as shown in part by the data in Table I A time sequence study of PCA reactivity and serum C' levels following C depletion as summarized in Table II again demonstrates the parallelism between these two events

For the experiments summarized in Tables I and II a polysaccharide immune system was used to elicit the cutaneous response and egg albumin rabbit anti egg albumin was used for the C' depletion procedure In other experiments similar results were obtained with other protein or pneumococcal antisera and their homologous antigens [19] The intravenous injection of 5 to 10 mg of zymosan also served to diminish C levels and suppress PCA [100]

Comparisons Between the in Vitro C fixing Potency of an Immune System and Its Capacity to Evoke PCA Reactions

Two examples may suffice to illustrate this relationship The first concerns the observation regarding the reduced C fixing efficiency of cross reacting immune systems as compared with the homologous reagents at equivalent weights of antibody [101 19] PCA studies in rats indicated that when SIII (specific capsular polysaccharide of the Type III pneumococcus) was used as the antigen to evoke a dermal response in rats the threshold level of homologous antibody N injected intracutaneously approximated 0 5 μg In contrast more than 10 times as much of the

TABLE II EFFECT OF AN IN VIVO IMMUNE REACTION ON LEVELS OF SERUM C' AND PCA RESPONSIVENESS

Antibody injected intravenously 200 μg rabbit anti Ea N (Serum 6456)
Antigen injected intraperitoneally 80 μg Ea N

Time in Hours After Ea anti Ez Injections	Average $C H_{50}$ per ml	Rabbit Anti SIII N (R_3–175) Injected Intradermally μg per skin site*			
		10 5	3 5	1 2	0 4
		Average Response mm †			
0	41 (8)*	18 (4)	14 8 (4)	8 3 (4)	5 (4)
0 25	24 (6)	16 (3)	6 (3)	2 7 (3)	0 (3)
0 75	>4 (4)	8 (4)	5 (4)	0 (4)	0 (4)
2 0	9 (4)	2 8 (16)‡	1 6 (16)	0 3 (16)	0 2 (16)
4 0	25 (4)	21 (3)	15 (3)	12 (3)	6 (3)
6 0	26 (8)	16 (3)	17 (3)	4 (3)	3 (3)

PCA reactions provoked with 24 μg SIII
† Figure in parenthesis indicate the number of rats for each determination Different animals were used for C and PCA estimations
‡ The results of several experiments are combined In many instances the PCA reactions are completely abolished under these experimental conditions

cross reacting antibody N was required when the heterologous antigen SVIII was used Thus for the serum R_3–164 which contained 1 93 mg anti SIII N per milliliter PCA reactions could be elicited with 0 1 ml of a 1 400 dilution of the antiserum (0 48 μg homologous antibody N) when SIII was used as the antigen The same serum contained 0 210 mg of anti SVIII N and 0 1 ml of a 1 4 serum dilution (5 μg cross reacting antibody N) was required for minimal PCA responses with SVIII as the eliciting polysaccharide The homologous reagents were also strikingly more efficient in *in vitro* C fixation assays

The second illustration along these lines concerns the interaction of rabbit antiribonuclease serum with three enzyme preparations, (*a*) the native enzyme used as antigen that is ribonuclease (*b*) the acetylated derivative and (*c*) the guanidinated derivative The data in Table III

TABLE III Comparison of PCA Responses and C′ fixing Potency of Rabbit Antiribonuclease (Serum 172–1) Reacting with the Native and Modified Enzyme Preparations

Antigen N Injected Intravenously	μg	PCA Reactions Rabbit Antiribonuclease N μg per skin site 12 6	6 3	3 1	1 6	Maximal C′H_{50} Fixed at 0 C with 2 5 μg Anti ribonuclease N
		Average Diameter of Skin Response mm				
Ribonuclease	30		14 8 (13)*	11 0 (13)	7 0 (13)	
	15		11 0 (13)	7 0 (13)	5 1 (13)	81
	5		0 (6)	0 (6)	0 (6)	
Acetylated ribonuclease	120	5 (3)	3 (3)	3 (3)	1 (3)	
	60†	1 (3)	1 7 (13)	0 (13)	0 (13)	14
	30		0 3 (13)	1 2 (13)	1 (13)	
Guanidinated ribonuclease	30	14 6 (11)	6 1 (11)	5 (11)	3 2 (11)	70
	15	5 4 (11)	2 5 (11)	1 (11)	0 (11)	

* Figure in parenthesis indicates the number of animals used for each determination

† One rat showed slight blueing at each skin site

compare the potencies of each of these antigens with the same antiserum in C′-fixation tests as well as in PCA studies The concurrent variation in these two properties emerges from these data as well as from those discussed immediately above and others presented by Osler *et al* [100]

3 *Antigen antibody Relationships in PCA and in C fixation*

One aspect of the systematic investigation of the immunological factors concerned in PCA relates to the quantitative interdependence of antigen and antibody A study of this relationship is summarized in Table IV and Figure 1

TABLE IV PCA RESPONSES IN NORMAL RATS WITH VARYING QUANTITIES OF RABBIT ANTI SIII N (R_3–175)* REACTING WITH SIII † DISSOLVED IN SALINE

SIII in Saline Intravenously µg	Rabbit Anti SIII N Injected Intradermally µg per skin site					
	10.5	5.3	3.5	2.6	1.2	0.4
	Average Diameter of Response in mm					
1.5	0.4 (8)‡		0 (8)		0 (8)	9 (8)
3.0	1.4 (22)		2.2 (22)		1.1 (22)	0.4 (22)
6.0	8.5 (57)		6.3 (57)		3.2 (57)	1.7 (57)
12.0	13.8 (56)		10.8 (52)		5.8 (52)	2.1 (48)
24.0	18.1 (33)		14.8 (25)		9.3 (29)	5.1 (25)
48.0	21.5 (4)	22.8 (4)		18.5 (4)	14.8 (4)	
96.0	18.5 (4)	14.8 (4)		14.0 (4)	8.8 (4)	
192.0	19.5 (4)	15.5 (4)		14.5 (4)	11.0 (4)	

* This serum was obtained through the courtesy of the New York State Department of Health and contained 5.26 mg anti SIII N/ml after absorption with the "C" substance of Type II pneumococci

† We are grateful to Dr M Heidelberger for the type specific pneumococcus polysaccharides used in these studies

‡ Figure in parenthesis indicates the number of rats used for each determination

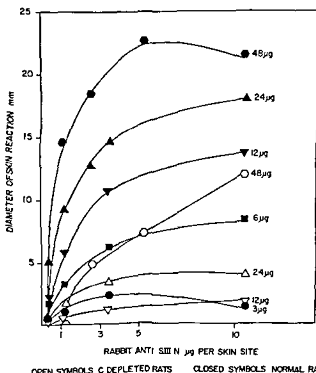


FIGURE 1 PCA reactions in normal and C depleted rats following challenge with varying amounts of SIII

It is apparent from these data as well as from those obtained in a similar study in guinea pigs[10] that a PCA reaction of uniform intensity may be elicited with one level of antibody and a corresponding amount of SIII or with a greater quantity of antibody with less antigen. Similar relationships for PCA have been obtained with protein antigens and homologous rabbit antisera in this laboratory and by Dr Ishizaka and his co-workers in experiments of the Schultz Dale type.[65] This reciprocal relationship between antibody and antigen applies equally well to the wheal and flare reactions in humans to pollen antigens as recently discussed by Sherman.[1 0] It is also common experience in tuberculin skin tests since an individual of lesser sensitivity requires a greater quantity of tuberculin for a positive reaction of the delayed type.[21] Although alternative interpretations of these observations may also apply, it should be noted that the quantitative aspects of the antigen antibody interaction in PCA are quite consistent with the hypothesized model which envisages the PCA response as an *in vivo* C fixation reaction wherein the tissue fixed antibody reacts with the parenterally introduced antigen in the presence of and possibly with the participation of C supplied by the host.

4 *Cation Requirements in Immune Hemolysis and Allergic Reactions of the Immediate Type*

In 1946 data were presented which described the enhancement of the lytic properties of C by Ca^{++} and Mg^{++}.[78] The role of these cations in the several reaction sequences which comprise immune hemolysis has been detailed by Levine *et al*.[6 66] and summarized in Mayer's studies.[14] With this background of information on the metal requirements for C function, efforts were undertaken to study the role of these divalent cations in allergic reactions of the immediate type. These attempts have met with only limited success. For example, the intraperitoneal injection of the sodium salt of ethylene diaminetetraacetate obliterated with PCA reaction in the albino rat. However, neither the calcium nor magnesium salts of this chelating agent had the same effect at concentrations equivalent to that of the sodium salt. While these results coincide with those of the *in vitro* hemolytic studies, the concentration of sodium salt required to produce this effect was but half of the concentration which was toxic to the rat. It is of interest, however, that similar results were obtained with citrate in the absence of toxic effects. In humans the appearance of wheal and flare reactions has been delayed for approximately 10 minutes when the offending pollen extract was diluted in sodium versenate. This effect was not observed with the calcium salt used as a control.[104]

The capacity of anions like citrate and oxalate which chelate the divalent cations to inhibit various immunological models of the allergic system has been recorded by numerous investigators for mast cell degranu

lation[84] anaphylatoxin formation[118] release of histamine from platelets[5] minced lung tissue[8] rabbit erythrocytes[43] and from human blood by the addition of specific pollen[81] Rocha e Silva has also reported the beneficial effects of citrate in contact dermititis

5 *C′ Component Relationships and PCA*

The demonstration that PCA responses were suppressed by C depletion stimulated attempts to restore the cutaneous reaction by means of C replacement These efforts were successful provided that the decomplementation procedure was unaccompanied by evident shock This was

TABLE V RESTORATION OF PCA REACTIONS IN C DEPLETED RATS

SIII per Rat µg	SIII Injected i v with 1 ml of	Rabbit Anti SIII N µg per skin site 10 5	3 5	1 2	0 4
		Average Diameter of Skin Response mm			
6	Isotonic saline	0 (8)*	0 (8)	0 (8)	0 (8)
6	Fresh guinea pig serum	8 6 (12)	7 2 (12)	5 1 (12)	3 (12)
6	Heated guinea pig serum (56° C 60 min)	2 5 (4)	2 5 (4)	0 5 (4)	3 (4)
12	Isotonic saline	1 8 (12)	1 1 (12)	0 4 (12)	0 3 (12)
12	Fresh guinea pig serum	3 5 (20)	4 1 (20)	1 8 (20)	0 5 (20)
12	Heated guinea pig serum (56 C 60 min)	4 (8)	4 5 (8)	2 5 (8)	0 8 (8)
24	Isotonic saline	4 3 (34)	3 5 (22)	1 4 (34)	0 4 (22)
24	Fresh rat or guinea pig serum†	10 4 (44)	6 6 (20)	3 9 (32)	0 5 (20)
24	Heated serum‡ (56 C 15 min)	10 5 (11)	5 8 (11)	3 7 (11)	2 (11)
24	Heated serum‡ (56 C 60 min)	5 5 (14)	4 9 (14)	2 4 (14)	0 7 (12)
24	Ammonia treated rat serum	9 9 (8)	7 5 (8)	6 0 (8)	0 (8)
24	Zymosan treated rat serum	3 9 (7)	2 1 (7)	1 4 (7)	0 (7)
24	Decomplemented serum§	6 1 (12)	4 2 (12)	1 5 (12)	0 (12)

* Figure in parenthesis indicates the number of animals used for each determination
† The injection of fresh guinea pig serum in one group of these rats increased the number of C H_{50} from a mean value of 9 to 37 per milliliter
‡ Sera from rats or guinea pigs yielded essentially similar results
§ Serum treated with washed specific precipitate (200 µg of rabbit antibovine gamma globulin N plus 24 µg of homologous antigen N per milliliter of serum)

achieved by injecting the antigen intraperitoneally and the antibody intravenously The union of these specific reactants and the resulting fixation of C occurred more gradually under these circumstances than when both reagents were injected intravenously and thereby facilitated restoration of the PCA in C deficient rats as shown in Table V

The skin reactions were also enhanced in normal rats when these were injected with hemolytically active serum or with serum preparations which were treated for preferential destruction of one or more of the

four C′ components, as seen in Table VI In the interpretation of these results, it should be recalled that the current procedures for preparation and estimation of C′ components are subject to several serious limitations The reagents described in Tables V and VI are not reproducible in a strictly quantitative sense Nor are the destructive treatments employed specific for a single component For example treatment of fresh serum with zymosan destroys components other than the third as observed by Pillemer *et al*[10] for human serum and in this laboratory for both rat and guinea pig serum As a consequence of these restrictions the results obtained with these reagents are considered as providing essentially qualitative evidence directed towards the provisional identification of a host factor which participates in PCA of the albino rat and are perhaps comparable to those reported by Kulka[59,60] It was considered that the

TABLE VI Effect of C′ Component Reagents on the PCA Response to Normal Rats

24 µg SIII Injected Intravenously with 1 ml of	C′ Components Present*	Rabbit Anti SIII N(R_1 – 175) Injected Intradermally µg per skin site 10.5	3.5	1.2
		Average Response in mm†		
Isotonic NaCl	none	11.2	6.2	0
Fresh guinea pig serum	1 2 3 4	23.5	16.2	10.0
Ammonia treated guinea pig serum	1 2 3	27.5	14.8	11.8
Specifically de C′ guinea pig serum	3	20.5	13.8	7.5
Fresh rat serum	1 2 3 4	18.8	14.2	11.5
Heated rat serum (56° C 15 min)	3 4	20.0	8.2	6.0
Zymosan treated rat serum	1 2 4	11.0	2.5	0

* As determined by the method described in reference 12

† Four rats used for each determination

demonstration of a requirement for one of the C′ components might be taken as evidence that the entire C′ complex participated in this reaction. The data summarized in Tables V and VI can be interpreted to imply that all the serum preparations which contained the third C′ component also possessed the capacity to enhance PCA

The numerous correlative lines of evidence relating PCA and C′ have been taken to indicate that the outcome of allergic reactions of the immediate type may be influenced by a host factor other than either antigen or antibody As discussed earlier serious difficulties beset any attempts to definitively identify a host tissue or serum constituent as hemolytic C′ The evidence presented in this section provides strong indications that the host factor may indeed be C′ but other interpretations

have not been eliminated Neither has it been possible to deduce whether C′ as a supposed participant is an essential mediator of PCA or serves merely to potentiate this reaction as has also been reported for specific precipitation [12, 83]

COMPLEMENT AND ANAPHYLATOXIN

In an effort to circumvent some of the difficulties which are indigenous to animal experimentation a different approach was undertaken to clarify the role of C′ in anaphylactic phenomena For this purpose recourse was had to the phenomenon of anaphylatoxin which has been studied by Friedberger,[36] Bordet [4, 5] Jobling [54] Bronfenbrenner [8] Novy and de Kruif [97] and in recent years by Rocha e Silva [120, 122] Anaphylatoxin it may be recalled is the name given to a histamine releasing substance produced in serum or plasma by a variety of substances including bacteria agar and immune aggregates It is readily apparent that studies confined to this phenomenon may suffer from the limitation that not all the important events in anaphylaxis are under experimental scrutiny Nor has it been uniformly accepted that anaphylatoxin is in fact an important manifestation or an integral part of the allergic reaction of the immediate type [14] Notwithstanding these considerations studies of anaphylatoxin provide an opportunity to effect a physical separation between those events which result in the production of a tissue damaging factor(s) and their effect on animal tissues In this manner the elucidation and characterization of some of the individual steps in the complex series of sequential reactions may be expedited Moreover these events can be investigated in a medium free of any cells or formed blood elements The experimental technique is relatively simple in that immune aggregates or suitable substitutes are reacted with fresh guinea pig or rat serum In our studies the consequences of this reaction are analyzed for (*a*) loss in hemolytic activity of the fresh serum (*b*) loss in activity of the third C′ component and (*c*) generation of a substance(s) which enhances capillary permeability and contracts smooth muscle

The data in Table VII summarize some of the recent findings of this laboratory on anaphylatoxin In one sense these data confirm many of the published reports concerning the appearance of anaphylatoxin or enhanced capillary permeability following the *in vitro* treatment of fresh hemolytically active serum with the reagents shown in Table VI or the *in vivo* administration of antigen-antibody [34] agar,[4, 7, 4, 46, 47, 52, 69, 70, 87, 93, 119] starch and dextran [0, 36, 4, 2, 67, 0, 7, 8, 88, 9, 97, 115, 117, 1, 1, 12, 1, 142] inulin [47, 78, 89, 93, 2] and yeast cells [13, 85] Perhaps the most striking conclusion supported by the data in Table VI indicates that each of the substances used by earlier investigators for anaphylatoxin production is destructive

of C′ The anticomplementary properties of dextran have recently been observed[137] If then the enhanced skin blueing may be equated to anaphylatoxin the correlation between the fixation of C and the genesis of a tissue damaging substance has been strengthened Thus tissue damage as indicated by increased skin blueing has not been observed in these experiments in the absence of C destruction Further validation of this finding would imply that the diminution of hemolytic

TABLE VII COMPARATIVE STUDIES OF C FIXATION C′3 UTILIZATION AND SKIN BLUEING ACTIVITY

Treatment of Rat Serum*	Temp C	Per Cent C′ Fixed C′H₅₀	Per Cent C′ Fixed C′3	R^2 at C′ = 1/75‡ mm
20 µg a Ea N†	37	79	27	16
100 µg a Ea N†	37	92	46	22
Refrigeration	4	59	9	16
Agar	37	91	86	28
Inulin	37	92	76	38
Dextran #1 NRRL	37	32	2	16
Dextran #8 CSC	37	51	41	34
Liquoid	37	95	79	30
Zymosan	0	64	0	22
Zymosan	15	62	32	32
Zymosan	37	76	81	48

* 2 5 ml of fresh rat serum (absorbed twice with sheep red cells) was treated with the designated reagent for 90 min at 37 C

† Used 4 ml of rat serum and an equivalent quantity of antigen

‡ Area of blue spot at a 1 → 75 dilution of C expressed as the square of the radius In other experiments values of R^2 as high as 50 have been obtained with agar Base line values may be as low as 6

potency of fresh sera is one of the processes which must necessarily precede or coincide with capillary permeability enhancement However the data in Table VI seem to suggest that C destruction may be an essential but not the sole or sufficient event leading to tissue damage Thus mere refrigeration of rat serum for o hours exerts a destructive influence as does one of the dextran preparations Yet in these cases capillary permeability was not potentiated Immune aggregates added to fresh serum at refrigerator temperatures likewise cause a reduction in the lytic potency of C without inducing much skin blueing It would appear that at least two other conditions must be fulfilled before a tissue injury of this type may be observed These are temperatures approaching 37 C and loss in C 3 activity The temperature effect confirms the finding of others[4 82 10] The possible requirement for C 3 destruction emerged as a finding of rather special interest in its apparent confirmation

of the observations discussed earlier on the relationship of this component to PCA

The data in Figures and 3 present a more detailed study of the action of agar and of antigen antibody on fresh rat serum in terms of C' fixation disappearance of C 3 activity and potentiation of capillary permeability by the products of the reaction(s) With regard to the action of immune aggregates (Figure) it may be noted that losses in hemolytic potency are observed prior to diminution in C 3 activity a finding long known to students of C' and which has recently been given theoretical clarification [74] Enhancement of capillary permeability is initiated at about the same level of rabbit antiovalbumin N which shows detectable destruction of C 3 on interaction with ovalbumin

The parallelism of these two events may be yet another coincidence or may merely reflect the sensitivity of the several techniques In our hands the C 3 titration developed by Rapp is equivalent if not superior to the quantitative C fixation technique in detecting minute losses of activity The skin blueing technique cannot of course be compared with these *in vitro* procedures The data for the agar treatment of rat serum resemble those for ovalbumin antiovalbumin system in that the capillary permeability effect appears more closely related to loss of C 3 activity than to the overall destruction of lytic potency In the case of

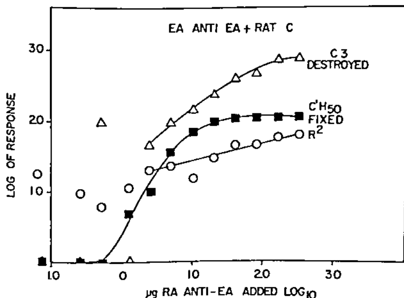


FIGURE 2

agar however the three activities parallel each other more closely than do the data in Figure .

The reasons for these differences are not apparent at this relatively early stage of investigation Several possibilities present themselves Thus studies of immune hemolysis by Rapp [110] and by Amiraian *et al* provide evidence that there may be more than a single compound in the preparations previously known as C'3 These data extend the findings made by Costa Cruz more than twenty five years ago [1] These demonstrations emphasize the need for more extensive studies of the type summarized in Table VII since it is entirely possible that specific precipitates affect the third component(s) in a different manner than do agar dextran and other preparations Another alternative of considerable interest suggested by the data in Figure 3 concerns the possibility that some substances for example liquoid or agar may utilize the third component by a mechanism other than that requiring utilization of C'1 C'4 and C' as in immune hemolysis Whichever if any of these possibilities will survive the rigors of further investigation it is more than likely that experiments designed for this purpose may serve to clarify further the role of C' in anaphylatoxin [41] and in allergic reactions of the immediate type

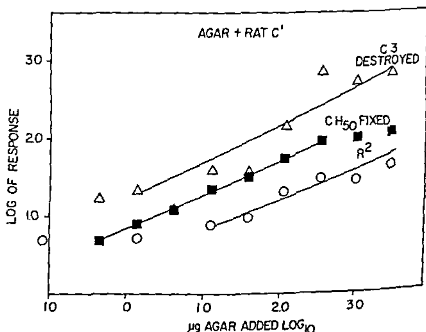


FIGURE 3

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In Vitro Models for the Allergic Reaction

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In discussing *in vitro* models for the allergic reaction, it is, of course, first necessary to specify what type of allergic reaction is being referred to as well as which models. In the following discussion only the immediate or anaphylactic (the terms will be used interchangeably) type of allergic responses will be meant when the term "allergic" is used, thus specifically excluding the large and important field of the delayed hypersensitivities and the Arthus reaction. The models to be discussed are (1) immune hemolysis, (2) the permeability factor of Miles and Wilhelm,[1] (3) the addition of peptone to serum, (4) the formation of serotoxin or, as it has been termed, anaphylatoxin, and (5) the release of histamine from the isolated tissues of sensitized animals.

The various models will be discussed from the viewpoint of the enzyme activation theory of the allergic reaction. The notion that the activation of an enzyme is involved in the genesis of the allergic reaction is an old one going back to at least the first decade of this century. Attention has usually concentrated on the activation of a proteolytic enzyme in serum or tissues, the theory being that the activated enzyme damages the tissues, causing the signs and symptoms directly, or indirectly by means of the release of pharmacologically active substances such as histamine, serotonin, heparin, etc.

Numerous workers have concerned themselves with the release of proteolytic enzyme activity during the course of the allergic or various model reactions. Nevertheless, there still remains room for doubt as to the relationship of the proteolytic activity found to the allergic reaction.[2] The most satisfying sort of evidence would be the isolation of the supposed enzyme (or more probably enzymes) in the requisite state of purity and the demonstration that these enzymes are capable of being activated by the antigen under conditions similar to those obtaining in the *in vivo* allergic reaction. In addition, it would be necessary to show that the activated enzyme is capable of yielding the same manifestations when injected (under the proper conditions of localization, etc.) into the unsensitized animal as does the injection of antigen in the sensitized animal.

The *amount of enzyme injected, of course would have to be similar* to the amount believed to be released during the antigen antibody reaction Merely to state this ideal is to indicate how very far we are from it In lieu of this sort of proof however one approach is to examine various models of the allergic reaction and see if they offer any evidence supporting the above or any other theory

Immune Hemolysis

The first model to be discussed is that of immune hemolysis or really the mechanism of complement action In doing so we will avoid the question taken up by Dr Osler as to whether complement is involved in the allergic reaction and merely stress its suitability as a model for that reaction In immune hemolysis one has cell damage manifested by the release of hemoglobin subsequent to and consequent upon the antigen antibody reaction occurring upon the cell This cell damage is not of course engendered by the antigen antibody reaction itself but by the antigen antibody reaction triggering off the complement system In the immediate type allergic reaction one also has cell damage manifested by the release of heparin, histamine serotonin, etc subsequent to and consequent upon the antigen antibody reaction occurring in the tissues From the work of Schild and Mongar and that of Osler *et al* the evidence is clear cut that just as immune hemolysis involves complement in the immediate type allergic reaction, something more than the antigen antibody reaction is also required

The present status of our knowledge of complement has been just reviewed by Dr Lepow He has summarized the evidence that following the antigen antibody reaction the initial event in immune hemolysis is the activation of the first component of complement from the precursor state *in which it exists in serum to an active enzyme* bound to the sensitized cell This enzyme is capable of splitting tosyl L arginine methyl ester (TAMe) benzoyl L arginine methyl ester and acetyl L tyrosine ethyl ester Part of the evidence for this hypothesis was the finding that di isopropyl fluorophosphate (DFP) is capable of inactivating C'1 and that this inactivation is prevented by the substrate TAMe[2] The significance of these observations is that DFP under the proper conditions has been shown to be a specific inhibitor of esterases Moreover it inhibits by attaching to active site of the enzyme in a sense it acts like a substrate which can t let go Thus the prevention by TAMe of the inactivation of C'1 by DFP can be considered as a competition for the active site of the enzyme This is illustrated in greater detail than in the previously published work by the data of Tables I and II In Table I is shown the hydrolytic activity of C'1 against a number of amino acid esters The acti

TABLE I SUBSTRATE SPECIFICITY OF C'1 ELUTED FROM LAC'1 4 2

Amino Acid Ester	Activity (μM acid produced)
1 Tosyl L arginine methyl ester (TAMe)	4 6
2 Benzoyl L arginine methyl ester	2 1
3 Acetyl L tyrosine ethyl ester	1 9
4 L Leucine ethyl ester	0
5 L-Lysine ethyl ester	0
6 L-Phenylalanine ethyl ester	0
7 DL Serine ethyl ester	0

Activity determined by means of the formol titration after 2 hour incubation at 37 C and pH 8 1 Lysine ethyl ester activity was tested both at pH 8 and pH 6 5 by means of the Ablondi copper phosphate technique The concentration of substrates was 0 02 M in all cases except lysine ethyl ester whose concentration was 0 04 M Acetyl L tyrosine ethyl ester was run in the presence of 5 per cent methyl cellosolve

TABLE II ACTIVITY OF AMINO ACID ESTERS IN PREVENTING INHIBITION OF C'1 BY DI ISOPROPYL FLUOROPHOSPHATE (DFP)

Amino Acid Ester		Inhibition of C'1 Activity
1 No DFP		0
2 DFP (5 0 × 10⁻⁴ M)		93%
3 Tosyl L arginine methyl ester (TAMe)	+ DFP	30%
4 Benzoyl L arginine methyl ester	+ DFP	68%
5 Acetyl L tyrosine ethyl ester	+ DFP	77%
6 L Leucine ethyl ester	+ DFP	91
7 L Lysine ethyl ester	+ DFP	93%
8 L Phenylalanine ethyl ester	+ DFP	91%
9 L Serine ethyl ester	+ DFP	90%

All amino acid esters 0 01 M in concentration The hemolytic C'1 activity determined by means of an R1[2]

vated C'1 was prepared by elution from sensitized cells containing the first second and fourth components of complement with ethylene diamine tetraacetic acid As can be seen only TAMe benzoyl L arginine methyl ester and acetyl L tyrosine ethyl ester were hydrolyzed under the conditions used

In Table II these same amino acid esters were tested for their ability to prevent the inactivation of C'1 on the sensitized cell by DFP The C'1 activity was decreased by 93 per cent when incubated with $5\ 0 \times 10^{-4}$ M DFP for one hour at 37 C The C'1 activity was tested for with an R1 after thorough washing of the cells The incubation of the same concentration of cells and DFP in the presence of 0 01 M TAMe benzoyl L arginine methyl ester or acetyl L tyrosine ethyl ester

gave only 30 per cent 68 per cent and 77 per cent inactivation respectively. *The presence of the other esters did not affect the extent of* inhibition by DFP

It is clear that only those esters which are substrates for the enzyme are capable of preventing the inhibition of C 1 activity by DFP It is also of some interest that the order of activity of the esters as substrates is the same as their order of activity as preventors of DFP inhibition This nice quantitative agreement might of course be coincidental the prevention of DFP inhibition is a measure of the affinity of the ester for the active site *while its activity as a substrate depends on other factors as well*

Granted that C 1 is an esterase does it act in immune hemolysis as an esterase and does it act directly to damage the cell? In regard to the first question while there is as yet no direct evidence bearing upon this point I have previously expressed my belief that C 1 is one of the large group of proteolytic enzymes which have esterase activity and that it functions *in immune hemolysis by virtue of being a proteolytic enzyme* In regard to the second question unpublished observations lead me to the belief *that the activated C 1 does not act upon the sensitized cell* other than being bound but acts upon C either directly or through C 4

Thus in this model we have the picture of the antigen antibody reaction activating an esterase capable of splitting TAMe as well as other esters and this esterase permitting other components of the system to complete the cell damage

THE PERMEABILITY FACTOR OF MILES (PF dil)

In 1955 Miles and Wilhelm showed that dilution of guinea pig serum 1 100 or more with physiological saline yielded an agent capable of increasing capillary permeability when injected into the skin of a guinea pig The permeability increasing factor they named 'PF dil They also showed that the activity of PF dil could be inhibited by very small concentrations of soybean trypsin inhibitor They therefore suggested that PF dil was a proteolytic enzyme even though they could show no direct correlation of proteolytic activity with PF/dil activity during the course of purification [1]

It is known that soybean trypsin inhibitor prevents the action of trypsin by combining with the active site [4] suggesting the possibility that the active site of PF dil is similar to that of trypsin The knowledge that trypsin is an esterase inhibitable by DFP suggested the trial of DFP on PF/dil in the presence and absence of various amino acid esters In collaboration with Dr Frank Austen such an experiment was set up using a purified preparation of PF/dil furnished by Dr Wilhelm The PF/dil was incubated at 37° C with 5×10^{-4} M DFP in the presence and

absence of 0 01 M concentration of various amino acid esters At the end of an hour the various mixtures were transferred to dialysis sacs and dialyzed in the cold against buffer (0 15 M saline 0 0 μ PO4 pH 7 6) overnight to remove the esters and the DFP As described by Miles the activity was tested by observing the diameter of the blue spot arising after injection of the PF/dil into the skin of guinea pigs which previously had been injected intravenously with trypan blue The results seen in Table III are the averages from 4 animals Miles considers that only reactions

TABLE III EFFECT OF AMINO ACID ESTERS ON INHIBITION OF PERMEABILITY FACTOR (PF/dil) BY DFP

Treatment of (PF/dil)	Diameter of Blueing (mm)
1 No DFP	9 6
2 DFP	3 1
3 DFP + Tosyl L arginine methyl ester (TAMe)	9 9
4 DFP + Benzoyl L arginine methyl ester (BAMe)	10 6
5 DFP + Acetyl L tyrosine ethyl ester	3 1
6 DFP + L Lysine ethyl ester	3 2
7 DFP + L Leucine ethyl ester	4 3
8 DFP + L-Phenylalanine ethyl ester	5 7
9 DFP + DL Serine ethyl ester	4 1
10 DFP + L Tyrosine ethyl ester	4 6
11 Saline control	1 7

5 γ of PF/dil injected into each site after removal of DFP and ester by dialysis All esters were 0 01 M and DFP 2 5 × 10^{-4} M Results are averages from 4 animals.

over 6 mm are significant and this was followed in evaluating the reactions The untreated PF dil gave a diameter of blueing of 9 6 mm which was brought to insignificant levels by treatment with DFP PF/dil incubated with DFP in the presence of TAMe or benzoyl L arginine methyl ester (BAMe) was essentially as active as the control with no DFP The other amino acid esters were incapable of preventing DFP inhibition of the permeability increasing activity

These results strongly suggest that PF/dil is an esterase capable of splitting TAMe and BAMe and thus provide powerful corroborative evidence for Miles and Wilhelm's hypothesis that PF/dil is a proteolytic enzyme

PEPTONE TREATMENT OF GUINEA PIG SERUM

It has long been known that the intravenous injection of peptone into animals produces a syndrome which is very similar to anaphylactic shock

It is therefore of interest in this connection that the addition of peptone to guinea pig serum increases the TAMe esterase activity of guinea pig serum As can be seen from Table IV (taken from unpublished work by Drs Austen, Marcus and myself) when peptone (proteose peptone Difco) *is added to guinea pig serum in the proportions of 100 mg/ml* and the mixture incubated for 5 minutes a significant increase in TAM esterase activity results compared to the control incubated without peptone Present evidence reviewed elsewhere[1] suggests the possibility that the increase in activity when the assay is conducted at pH 8 0 is due to the partial activation of C 1 by peptone The increase manifested at pH 9 0 is believed due to another enzyme not C'1 and probably not one of the plasminogens[1]

Here again we have evidence that activation of TAMe esterase occurs although of course we have no evidence that the activation of these

TABLE IV THE ACTIVATION BY PEPTONE OF SOME TAMe ESTERASES OF GUINEA PIG

	Activity (μm acid/ml serum/30 min)	
	pH 8 0	pH 9 0
Untreated serum	28	14
Peptone treated serum	48	26
Increment due to peptone treatment	20	12

Peptone and serum in the proportions of 100 mg proteose peptone Difco ml serum incubated 5 minutes at 37 C before assay Assay was carried out at 37 C according to the technique of Troll and Sherry

esterases is causally connected with peptone shock The finding that C 1 is activated by peptone does suggest the possibility that peptone shock might result from the activation of the complement system This of course is at present merely a suggestion unsupported by any proof or real evidence

While the findings just described do demonstrate that TAMe esterases are activated by peptone they tell nothing of the relationship of these enzymes to the increase of proteolytic activity which has been reported by numerous workers to follow peptone addition to serum Using the fibrin plate technique of Astrup and Mullertz we were able to confirm Ungar's[11] and Olesen's[9] findings that peptone (proteose peptone Difco 100 mg ml serum) added to guinea pig serum activates a fibrinolytic enzyme This was demonstrated either by adding the peptone whole serum mixture to the plate or by adding the euglobulin prepared from the mixture essentially as described by Ungar The fibrinolytic activity

so obtained was not diminished by DFP in 0.01 M final concentration when the DFP was added to the whole serum either before or after the addition of the peptone and the mixtures allowed to incubate 2 hours at 37° C. Thus one can conclude that the increase in fibrinolytic activity is probably not due to the TAMe esterases activated by peptone. It is also probable that the proteolytic activity is not due to the activation by peptone of plasminogen to plasmin as assumed previously, if one defines plasminogen as the proteolytic enzyme(s) of human serum capable of being activated by streptokinase or the proteolytic enzyme of other species capable of being activated by streptokinase and human activator. Troll and Sherry have shown that the plasminogen so defined is an esterase which splits TAMe and lysine ethyl ester and we have confirmed this for guinea pig serum.[2] Dr. Marcus and I have shown that both the TAMe esterase activity and the fibrinolytic activity of the plasmin obtained from guinea pig serum activated by streptokinase and human activator are inhibited, as expected, by incubation for 90 minutes at 37° C with 1.0×10^{-2} M DFP.

Serotoxin or Anaphylatoxin

It has been known for some time that more than one serum component apparently takes part in the formation of serotoxin. Bordet showed that serotoxin could not be formed from heated serum, although it was later shown that serotoxin once formed was stable to such heating. On the basis of this temperature lability Rothschild and Rocha e Silva[6] suggested that the activation process might be enzymatic and made the further suggestion that serotoxin itself might also be an enzyme. The multiple nature of the serotoxin system has received confirmation and extension by the recent painstaking work of Hahn and Giertz.[3, 5]

In experiments carried out with Dr. Frank Austen, serotoxin was formed by treating rat plasma with agar as described by Rocha e Silva and its activity tested on the guinea pig ileum.[6] It was found that incubation of the activated rat plasma with 0.01 M DFP for as long as 1½ hours at 37° C did not appreciably decrease its activity compared with the control plasma incubated without DFP. DFP itself evokes a sustained contraction of the guinea pig gut. This contraction has a prolonged induction period and so no difficulty was experienced in differentiating this from the contraction due to serotoxin with its much shorter latent period. Thus it can be concluded that serotoxin is probably not an esterase, although the possibility of its being some other type of enzyme is not excluded.

As pointed out, it has been suggested that the activation of serotoxin might also be enzymatic. An attempt was made to see if the activation process involved a DFP inhibitable step by adding 0.01 M DFP to rat

plasma 5 minutes *before* the addition of the agar then the mixtures were incubated at 37° C for one hour In one experiment the activity was tested for directly in another experiment the activity was tested for following dialysis of the mixtures against 0 3 M saline overnight in the cold No real diminution in activity occurred in the rat plasma activated in the presence of DFP This was also true when in other experiments plasma diluted 1 10 was used as the source of serotoxin While it is tempting to conclude from these results that the activation step also does not include a DFP inhibitable step the temptation must, for the present at least be resisted It is possible that under the experimental conditions used the change of the activator from a precursor to an active state and its subsequent action on the precursor form of serotoxin might be much more rapid than the ability of DFP to inhibit the activator

In Vitro Histamine Release

In London in August of 1957 Dr John Humphrey of the National Institute for Medical Research informed me of unpublished work of Dr W E Brocklehurst of the same institution Dr Brocklehurst has very kindly given me permission to describe his findings If the lungs of sensitized guinea pigs were perfused with 0 013 M DFP and the DFP washed out before the addition of antigen a decrease resulted in the amount of histamine released compared with the control If however antigen was added in the presence of DFP the decrease of histamine released was considerably greater On the other hand DFP added 2 minutes after the addition of antigen had no effect The release of slow reacting substance (SRS A) more or less paralleled the changes in histamine release From this Dr Brocklehurst concluded that DFP inhibits a very early link in the chain of events [while the] inability to block completely is probably due to the sudden activation of ( ) the enzyme [involved in this link] which acts only for a very brief period subsequent links not being inhibited by DFP The speed with which this link is established and passed is such that inhibition can only be partial

These results are most suggestive The concentrations of DFP used are of course extremely high and one would like to see activity with lower concentrations of DFP However Dr Brocklehurst s explanation has experimental support from the observation that the same high concentrations of DFP are required to inhibit C 1 when diluted whole guinea pig serum (i e C 1 in the precursor form) is added to sensitized red cells even though much lower concentrations are effective when the C 1 is in the already activated state

In the models studied it is clear that (1) a TAMe esterase is involved in complement action ( ) PF/dil is a TAMe esterase (3) peptone is

capable of activating TAMe esterases of serum (this allows the possibility of a TAMe esterase being involved in peptone shock although the fibrinolytic enzyme activated by peptone is probably not an esterase) and (4) the evidence of Dr Brocklehurst is at least suggestive that an esterase might be involved in *in vitro* histamine release by an antigen antibody reaction It is also clear that serotoxin or anaphylatoxin is not an esterase The present evidence can be taken to indicate that an esterase is not involved in the activation of serotoxin reasons however have been given why further experimentation is necessary before this conclusion can be accepted

In those models in which there are indications that esterase activation plays a role it is of interest that except for PF dil there are also indications that the esterase is an early link in the chain of events In other words the esterase is not directly responsible for the tissue damage In the case of PF/dil it is likely that it is the enzymatic activity of the PF dil which is responsible for the increase of capillary permeability yet from the most recent work of Wilhelm and Miles it is also unlikely that PF/dil plays any role in anaphylaxis [13]

It has been assumed that the element common to all the models and to anaphylaxis itself is the ultimate activation of plasminogen to plasmin [3] If plasminogen is defined as was done in this paper it would appear from the work just described that plasminogen is not activated by either peptone or by agar In other work we so far at least have not been able to detect any effect of an antigen antibody complex on the plasminogens of guinea pig serum [3] This negative finding is in accord with the observations of McIntire that bovine plasmin is not able to release histamine from rabbit platelets and the observations of Tillot that the injection of purified streptokinase into humans does not lead to anaphylactoid signs or symptoms This of course is not to deny that other proteolytic enzymes might not be activated by these various agents it is merely to raise the question if a proteolytic enzyme (or enzymes) in serum is activated by these several substances and it is not plasminogen what is it? As yet we have no answer

Nevertheless the work just described does suggest the possibility that a general type of mechanism involving the activation of an esterase early in the course of events might be common to at least certain of the models and suggests (by a further flight from the evidence) the possibility that this same type of mechanism might be operative in the various forms of the immediate type hypersensitivity

The inability of the fibrinolytic enzyme activated by peptone to be inhibited by DFP while plasmin plasminogen the enzyme activated by streptokinase (or streptokinase and human activator) is inhibited reveals how complex the situation is even in this relatively simple model It pro

vides us with an example of the need for caution before we ascribe all reactions in the various models to the same enzyme system (complement or any other) and further assume that this same enzyme system is responsible for the various forms of the immediate type hypersensitivity Here as elsewhere the words of Seneca are much to the point We imagine ourselves initiated in the secrets of nature we are standing on the threshold of her temple (Quoted from J B Bury *The Idea of Progress*)

ACKNOWLEDGMENTS

I wish to thank Dr Bernard Jandorff of the Chemical Warfare Laboratories for his gift of the DFP Dr W E Brocklehurst of the National Institute for Medical Research for allowing me to describe his unpublished results Mr James W Eichelberger Jr of Walter Reed Army Institute of Research for performing the fibrin plate assays and Drs Miles and Wilhelm of The Lister Institute of Preventive Medicine for their gift of the purified PF/dil

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DESIGNATED DISCUSSION

Herbert J. Rapp (Baltimore, Maryland): I think the work of Dr. Osler on the role of complement in passive cutaneous anaphylaxis represents a major step toward eventual acceptance of the idea that complement may be a factor in the mechanism of hypersensitivity phenomena in experimental animals. As Dr. Osler has pointed out, however, both here and in his publications, it should be emphasized that at the present time the evidence for the role of complement in passive cutaneous anaphylaxis is correlative and not direct. Indeed, it may not be possible to settle this question by direct experiment until more is known of the fundamental nature and action of complement.

If Dr. Osler is correct about the role of complement in anaphylatoxin production, I wonder why Dr. Becker does not find inhibition of this phenomenon by di-isopropyl fluorophosphate. The concept that complement participates in allergic reactions as an enzyme or enzyme system is most interesting and represents a fruitful approach to the problem. I should like to join Dr. Becker, however, in urging extreme caution in extrapolating from *in vitro* to *in vivo* experience.

It would be most helpful if a substrate other than amino acid esters could be found which is associated with the biological phenomena. In this regard I think too little attention has been given to the role of the cell in hemolysis and hypersensitivity. Thus it is entirely possible that inhibitors such as DFP may not be directed against an enzyme associated with complement but perhaps a cellular constituent which is activated as a result of complement action.

As Dr. Lepow has pointed out, it cannot yet be categorically stated that C 1 is an esterase. It is possible that the esterase activity is not associated with C 1. Again I should like to emphasize that this is a situation in which we have an enzyme in search of a natural substrate.

In addition, I wish to point out that the intermediate product EAC 1,4 employed by Dr. Becker to demonstrate its TAMe esterase activity differs somewhat from the product described by Levine. The hemolysin used by Levine was prepared in rabbits by the injection of boiled sheep erythrocyte stromata and is therefore Forssman antibody. The hemolysin employed by Becker is the so-called commercial amboceptor prepared with whole sheep red cells and probably contains isophile as well as Forssman antibody.

With the Forssman antibody used by Levine, precise information was available concerning the number of antibody molecules required to achieve optimal sensitization of the cells. Unfortunately, this information is not available for commercial amboceptor because of the difficulty of

obtaining a weight analysis for isophile antibody However the concentration of antiserum employed by Becker to sensitize red cells was about five times as high as that employed by Levine

Finally EAC 1 4 2 prepared by the method of Levine at least in my hands, does not exhibit TAMe esterase activity However I was able to obtain esterase activity with EAC 1 4 2 prepared in Dr Becker s laboratory under his supervision and with his reagents It is not known yet whether this discrepancy represents a qualitative difference due to the different kinds of hemolysins or a quantitative difference due to the number of antibody molecules per red cell in the two different EAC'1 4 2 preparations

I believe that studies directed toward the implication of complement in hypersensitivity reactions will prove to be a fruitful approach While there are strong indications in favor of the enzymatic nature of complement it is difficult to evaluate the role of C 1 as a proesterase in the absence of a substrate or substrates which are involved in the biological reactions

OTTO J PLESCIA (New Brunswick New Jersey) My role as a discussant reminds me of an interpretation placed upon the word discuss by a professor of chemistry whom I had as an undergraduate He liked to phrase his questions Discuss so and so and to him the word 'discuss connoted the privilege to cuss and discuss

Fortunately I have no need to do any cussing as part of my job of discussing because the case for the possible participation of complement in allergic phenomenon has been admirably stated by our speakers In fact inasmuch as I concur by and large with their presentations and their conclusions it would be far simpler for me to say The defense rests However since there are a few minutes remaining it may not be repetitious to make a few remarks which would be more in the way of summation

First of all there is no need to point out to this audience the complexity of the phenomenon of hypersensitivity with all its manifestations The very fact that three days of symposium are required just to touch upon the various aspects of the problem speaks eloquently for this complexity We could easily stay here another three days and discuss the complement system itself without even talking about the possible involvement of complement in hypersensitivity Obviously if we couple one complex system with another the complexity of the complex rises exponentially and it would be easy for someone who might adopt a purely skeptical attitude to pick holes in the evidence which purports to show that complement may possibly be involved in allergic phenomena

Therefore I should like to urge that we take a more positive attitude

that we look at the data and accept them at face value draw conclusions with due restraint and caution examine the loopholes that exist and do our level best experimentally to plug the loopholes

What is the nature of the problem confronting us if we are to relate the involvement of complement to allergic phenomena? I think the problem may be stated by posing three questions

First in addition to the involvement of antigens and antibodies in allergic phenomena are there host components or factors involved If there are none our job is complete and we need go no further If the answer is in the affirmative then we must ask ourselves a second question What are these factors? Are they complement always sometimes or not at all If complement is involved at all the third question is What is the possible role—the mechanism—by which complement participates in allergic phenomena?

Regarding the first question I think there is little doubt that host factors besides antigens and antibodies do participate in allergic phenomena

As for the second question as to whether it is possible that complement does play a role in these phenomena I think Dr Osler has done an admirable job in selecting a particular manifestation of the allergic phenomenon which is susceptible to controlled experimentation As Dr Rapp has also pointed out the evidence that Dr Osler has obtained is not causal but is certainly correlative and is strongly suggestive that complement is probably involved at least in this particular instance of an allergic manifestation

It is unfortunate however that we cannot be more definite in our conclusions because our knowledge of complement is still incomplete and because we have not yet succeeded in isolating the various components of complement in pure form We are also limited in that the reactions in which complement participates are not so specific that we know that only complement is involved and no other components of serum This is true for both complement fixation and for the inactivation of complement by zymosan

Concerning the third question the mechanism of allergic reactions we note throughout the various model systems which Dr Becker has presented to us this morning and the discussion of Dr Schild last night a variation on the theme that the activation of enzyme systems and the subsequent action of these enzymes in intermediate steps play an important role Such a concept merits further attention in view of the characteristics of the complement system reviewed by Dr Lepow this morning It is not impossible and perhaps it is highly probable that in some instances at least, complement does fulfill the role of such enzymes

In conclusion I would predict that if we are to succeed in demonstrating and elucidating the host factors which participate in the intermediate steps that eventually lead to tissue damage and subsequent

characteristics of allergic manifestations and if we are to implicate complement causally, at least in some systems then we must probably await the time when we shall have pure components of complement that we can put into the system at will and when and where we want them. Also I think we must wait until we are able to take the allergic reaction from the upper stratosphere in the realm of animal biology and bring it down to the level of molecular biology closer to *terra firma*

GENERAL DISCUSSION

JACOB J PRUZANSKY (Chicago Illinois) *In relation to the role of complement in anaphylaxis* we have been working for some time on anaphylaxis in the guinea pig that is passive anaphylaxis of a general type with an aerosol challenge I wish to comment on our results and also on Dr Osler's work

First if we assume that complement acts in anaphylaxis we must assume that complement is destroyed in this action because up to the present time every known action of complement leads to some destruction or disappearance of C whether it is activation of esterase hemolysis or fixation

We know from perusal of the literature that the drop in complement in anaphylaxis as measured in the animal may be vanishingly small It may be almost nonexistent and so we may have to assume then if we *are to postulate a role for complement, that the amount of complement* necessary for the anaphylactic reaction may be vanishingly small

I cite Osler's data in his recent paper to substantiate this fact When the complement titer was reduced from 40 units to 9 as he did with a certain level of antigen antibody, he got a depletion of complement and a lack of hypersensitivity reaction However, if he increased the antigen he restored the reactivity I don't believe this fits well with the assumption of a role for complement in anaphylaxis It is, however compatible with the possibility that very small amounts of complement may be involved

I might also cite the fact that we have been doing histamine release studies in which complement may be involved and has been implicated In histamine release we have separated cellular elements of rabbit blood from the plasma incubated them with antigen antibody and obtained *histamine release These are not washed cellular elements* so there may be a residuum of complement although none may be measured by titration. I cite this as further corroboration of the fact that a vanishingly small amount of whatever serum factor is necessary may be acting

Using the decomplementation technique that Osler used which is essentially the same as the one which we have used in general anaphylaxis of the guinea pig we have found similar results to those of Dr Osler We

have inhibited the anaphylactic reaction by an antigen and an antibody that are extraneous to the system However I might say that in most instances we have been able to inhibit the anaphylactic reaction when we remove only one third of the total complement present This leaves two thirds still present in the circulation When you have two thirds of the total guinea pig complement you have a relatively large amount of complement still available in the circulation and if the number of units needed for reaction may be vanishingly small as I have just indicated we have a paradoxical situation indeed

We thought that we were probably dealing with a tissue reaction and not a serum complement reaction and therefore in addition to the described type of decomplementation we decomplemented by aggregates that is by antigen antibody aggregates This gives the same amount of destruction or fixation of complement as one obtains by the injection of antigen and then later antibody However this procedure does not inhibit the anaphylactic reaction that we have been studying This seems to indicate that the locus of activity is in the tissue and that circulating elements or events play only a minor role in the inhibition of anaphylaxis

I have one specific question The data in the paper by Dr Osler indicate if I remember the data properly maximal decomplementation at three quarters of an hour However there was little or no inhibition of PCA at this point whereas at two hours maximum inhibition of PCA occurred and complement was already being regenerated I wonder if Dr Osler could explain the time discrepancy between the point of minimum complement titer and minimum PCA reactivity

Chairman Lepow The comment by Dr Pruzansky about the difference between aggregates formed by separate addition of antigen and antibody and preformed specific precipitates is reminiscent of a situation which obtains in the properdin system In these experiments the separate addition of antigen and antibody to serum results in removal or inactivation of certain of the cofactors which are required for formation of the properdin zymosan complex However the addition of preformed aggregates in the same amount and ratio of antigen to antibody and under the same conditions of temperature time and so on does not result in the disappearance of these activities I am not suggesting a parallelism here but merely cite another case where preformed antigen antibody aggregates behave differently from antigen antibody aggregates formed in the presence of serum

Dr Osler I am glad that Dr Pruzansky has succeeded in confirming some of our findings thus providing a common basis from which to proceed

I am not certain of the meaning of the last point raised by Dr Pruzansky since the data presented here this morning are entirely consistent with those published previously There would appear to be several cogent reasons for avoiding the use of preformed immune aggregates in a complement depletion procedure It is well known that many nonimmune factors exert a profound influence on the outcome of the anaphylactic reaction Hormonal influences histamine and serotonin availability and factors of stress are only a few that come to mind at this moment The use of particulate material is probably attended with effects such as pulmonary capillary damage and other aspects of generalized shock which might well obscure experiments designed to study the release of histamine by antigen and antibody Therefore the failure to elicit PCA reactions despite the presence of moderately high levels of complement need not be pertinent to the problem at hand With respect to our own studies it was necessary to reduce to a minimum any symptoms of shock in complement depleted rats before successful restoration of the PCA reaction was achieved

The last comment to be made with respect to Dr Pruzansky s discussion about complement levels refers to the method of titration It may well be that if complement titrations had been performed by available spectrophotometric methods rather than by the usual serum dilution technique which is subject to a large experimental error our agreement might be closer than is presently apparent

WILLIAM J KUHNS (Pittsburgh Pennsylvania) I should like to ask Dr Lepow a question

Dr Lepow you indicated that a serum inhibitor or inhibitors exist for esterase activity Since there are variations in complement utilization in various diseases presumed to have antigen antibody etiology I wonder if you – or someone in your group – have had any experience with variations in such inhibitors in different diseases or if you think there is any difference in what the inhibitor might be in terms of molecular size or chemistry

CHAIRMAN LEPOW That is a very interesting question Dr Kuhns We are in the process of collecting sera from patients with a variety of diseases, including the collagen group to see if there may be differences in the inhibitor level If one is dealing with an enzyme which may play a role in hypersensitivity and if there is an inhibitor to this enzyme one must consider at least two possibilities Activity of the enzyme may be due to its activation by some exogenous means or by some mechanism which results in a decrease in inhibitor level so that autocatalytic activation can occur

As far as the physiochemical properties of the inhibitor are concerned there again work is in progress and there is very little to report of a definitive nature at this time We are actively engaged in the isolation of the inhibitor but the work is in a very early stage

DR SEHON Dr Lepow has stated that the activation energy for the reaction step leading to the activation of esterase is 31 kcal /mole How is the appropriate constant derived from such a complex system? Is this activation energy calculated from the overall rate of reaction? If so this would in all probability involve some rate determining step which might be what one measures kinetically However this step might not be identifiable with the activation process of the esterase

CHAIRMAN LEPOW We are not measuring the overall complement system here The energy of activation which was obtained — 31 kcal /mole — was obtained by measuring as a function of temperature the rate of disappearance of partially purified first component of complement which is free of other components At the same time that we measure the disappearance of hemolytic activity we measure the rate of appearance of esterase activity and the rate of appearance of complement inactivating activity each in separate assay systems It is quite possible to get rate constants, which are accurate within the limits of error of the assay procedures and it is quite possible to calculate an *apparent* energy of activation or critical thermal increment The relation of such a value to the true energy of activation is of course open to question

VI

Autoantibodies

Chairman FRANK L. ADLER, Ph.D. (*New York, New York*)

Chairman's Remarks

About sixty years ago Ehrlich rejected the possibility of autosensitization on teleological grounds. His concept was promptly challenged by the experimental production of autoantibodies reactive with spermatozoa or lens, the demonstration of autoantibodies against red cells, and by the suggested autoantibody nature of the Wassermann antibody. Evidence accumulated since then firmly supports the notion that the barrier envisaged by Ehrlich is not entirely impregnable. In fact, antibody reactive with extracts from various tissues, with certain red cell antigens, or with such simple body constituents as glycogen seems to appear with frequency in the sera of "normal" individuals though without apparent adverse effects. It has been suggested that the "horror autotoxicus" pertains only to those potential antigens which normally are in intimate contact with blood and lymph. A somewhat different concept, supported by recent work on acquired tolerance, assumes that contact of the potential antigen with antibody producing cells or their precursors during embryonic life is the factor which interdicts autoimmunization upon attainment of immunological maturity.

The validity of these two suggestions seems to be impaired, that of the former by the existence of autoantibodies against surface antigens of the formed elements of the blood, that of the latter by the finding that some of these autoantibodies are specific for blood group factors which are known to be present very early during embryonic development. Finally, there is the notion that autoantibody formation is a normal physiological process which assumes pathogenic importance only if excessively stimulated. This and the acquired tolerance concept share the merit of providing working hypotheses which with tools and methods now available can be tested experimentally.

The formation of certain autoantibodies can be stimulated by the injection of tissue preparations derived not only from the individual concerned but also from other individuals of the same or different species. The transcendency of organ over species specificity observed in such instances has provoked much speculation on the nature of the antigens involved and the character of the changes which might render a substance autoantigenic. These aspects of the problem are currently under investi-

gation in several laboratories. Any generalization on physical, chemical or immunological properties of such antigens as a class appears to be premature at the present.

The experimental production of autoantibodies, with or without associated lesions, succeeds regularly with only a few antigens. More common is the negative result obtained, for example, upon injection of Forssman antigen into an animal which contains this factor in its tissues. Another example is the reaction of the rabbit to the injection of bovine serum albumin. The antibody found free in the rabbit's serum reacts with bovine and other mammalian serum albumins but not with the serum albumin of rabbits. Lesions encountered in the course of such immunization are those of the serum sickness type and are attributed to the reaction between antibody and the foreign antigen.

Attempts to transfer experimental autosensitization passively, either *with serum or with cells from sensitized animals*, have thus far failed. There is much to suggest that conventional serum antibody may not be the noxious agent; even in hemolytic anemias clinical remissions may occur in the continued presence of such antibody in the serum and on the cells. The successful transfer of autosensitization between parabionts and its observed transmission from mother to embryos suggest, however, that past failures may have been of a technical nature. If and when such experiments succeed, we may finally learn whether the circulating autoantibodies are the cause or a result of some of the pathological conditions in which they are encountered.

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*The Incidence, Nature, and Significance of Auto-antibodies in Thyroid Diseases**

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(London England)

It has recently been established by Witebsky and his co workers [4, 41] that various animals can be immunized against their own thyroid tissue and that this is accompanied by the appearance of circulating antibodies and is associated with destructive lesions of the thyroid gland which are roughly proportional to the antibody level

Studies of serum proteins in human thyroid diseases [6, 7] led to the demonstration of similar autoantibodies in patients with various forms of chronic thyroiditis primary myxedema subacute thyroiditis of virus origin and thyrotoxicosis [8, 34]

A common histological feature in all these conditions is the presence of lymphoid and plasma cell infiltration of the thyroid gland either local as in thyrotoxicosis or diffuse as in chronic thyroiditis In this condition, the invading lymphoid tissue and constant efforts at regeneration on the part of the gland lead to the production of a firm goiter When the destructive process predominates it leads to loss of endocrine function and ultimate myxedema in the patient The size of the goiter varies from about half the normal thyroid weight of 25 Gm when the gland is palpable owing to its firm consistency to large goiters encircling the trachea and weighing up to 350 Gm The compensatory enlargement of the thyroid appears to be dependent on TSH overactivity and the ability of the gland to respond to it In primary myxedema lymphoid and plasma cell infiltration and circulating antibodies are often found but since there is no sustained effort at regeneration no new antigen is available to maintain the stimulus for lymphoid overgrowth and no compensatory enlargement of the thyroid gland ensues

The facts outlined above have led to the hypothesis that human thyroiditis is a manifestation of autoimmunity and that destruction of

*This work was supported by the research funds of the Middlesex Hospital Medical School and grants from the British Empire Cancer Campaign and the Will Edmunds Clinical Research Trust of the Royal College of Physicians

the gland in this and in primary myxedema is due to a progressive chain reaction between autoantibodies and their various antigens in the thyroid

We report here further studies on the characteristics of the human autoantibodies some of which have already been published[33] We have studied the occurrence and distribution of these antibodies in various forms of goiter by precipitin reactions complement fixation and tanned cell hemagglutination We will discuss what is known of the thyroid antigens and describe efforts made to separate and identify them

The clinical significance of the autoantibodies will be considered in the light of experience with patients and the results of immunological tests The possible factors which initiate and perpetuate destructive chain reaction will be discussed

Detection and Estimation of Thyroid Autoantibodies

Precipitin Test

The qualitative detection of precipitating antibody against human thyroglobulin was conveniently carried out using a double diffusion modification of Oudin's method of precipitation in agar gel columns[26] as described by Doniach and Roitt[8] Saline extracts of normal post mortem glands or of thyrotoxic thyroids obtained at operation served as antigens conditions were more clearly defined by using a purified thyroglobulin preparation (5 mg/ml) obtained from these extracts by ammonium sulfate fractionation[5]

Quantitative evaluation of the level of precipitating serum antibody was made by the method of Heidelberger and Kendall[18] Increasing amounts of thyroglobulin were added to a series of tubes containing a constant volume (0 1 ml) of serum after incubation at 37° C for 0 5 hr and storage at 2° C for 4 days the precipitates were spun down washed twice with ice cold saline and dissolved in 2 0 ml of 0 1 M sodium carbonate The protein content of the resulting solutions was estimated by measuring the optical density at 260 mμ. The following relationships were used in calculation of the antibody content of the precipitates $E^{0.5\,cm}_{260\,m\mu}$ 0 100 = 139 μg gamma globulin or 0 μg thyroglobulin The supernatants from the specific precipitates were tested for the presence of antigen and antibody by the ring test

Tanned Cell Hemagglutination Test

The method was substantially that described by Witebsky and Rose[40] with certain modifications Human O cells were used within 7 days of being drawn into citric acid dextrose solution A 4 per cent suspension of the washed cells in saline buffered at pH 7 was treated with an equal

volume of tannic acid (1 0 000) for 10 minutes at room temperature The tanned cells were gently washed once resuspended to give a 2 per cent suspension and coated with antigen at pH 7 by mixing with an equal volume of purified thyroglobulin solution (5 mg ml) After 30 minutes at room temperature, the cells were gently washed thrice with buffered saline containing 1 per cent normal rabbit serum (inactivated and absorbed with human O cells) and finally adjusted to give a 1 per cent suspension The coated cells (0 1 ml) were added to each serum dilution (0 1 ml) contained in a Perspex agglutination tray and the results read after 3 hours at room temperature and after standing overnight at C The readings were graded according to Stavitsky [38] and the end point taken as the highest dilution at which one plus agglutination was evident The titer is expressed as the reciprocal of the serum dilution Serial dilutions of serum were made with a single pipette when screening a large number of patients Although this procedure is subject to error at high dilutions a Hashimoto serum containing precipitins gave a titer of 1 000 000 when separate pipettes were used for each dilution The validity of the end point was confirmed by the finding that the minimum concentration of thyroglobulin required to inhibit agglutination by a 1 5 dilution of serum was inversely proportional to the highest dilution of serum giving positive agglutination As a control thyroglobulin (0 1 ml of a solution containing 25 mg /ml) was mixed with serum diluted 1 5 before addition of the red cells The results were not accepted unless the control was completely negative Nonspecific agglutination rarely occurred and could frequently be eliminated by prior absorption of the serum with O cells

Complement fixation Test

The micromethod of Belyavin [3] was used as adapted to thyroid work by Trotter Belyavin and Wadhams [40] The antigen was prepared by homogenizing thyrotoxic thyroid obtained at operation with 3 volumes of saline buffered with borate at pH 7 6 in a Waring blender for 3 minutes The homogenate was poured through muslin and spun at 3000 r p m for 15 minutes to remove large particles and the supernatant was kept at – 0° C until required for the tests Under these conditions complement fixing activity was maintained for several months Selection of the antigen was important for successful results Some thyroid glands were pro complementary but it was found that if the patient had complement fixing antibodies in her serum her excised thyroid gland was anti complementary presumably because the active protein was already combined with antibody the presence of tanned cell agglutinating anti body in the patient s serum did not interfere with complement fixing activity but we prefer to use glands from patients having no circulating

MRS B.T. ♀ 67 (My oede)

ANTIGEN DILUTIONS ⟶

SERUM DILUTIONS ↓

1	2	4	8	16	32	64	128	256	512	SERUM CONTROL
12	●	●	●	●	●	o	–	–	–	–
4	●	●	●	●	●	o	–	–	–	–
8	●	●	●	●	●	–	–	–	–	–
16	●	●	●	●	●	–	–	–	–	–
32	●	●	●	●	●	–	–	–	–	–
64	●	●	●	●	●	o	–	–	–	–
128	o	●	●	●	●	o	–	–	–	–
256	o	o	o	o	o	o	–	–	–	–
512	–	–	–	–	–	–	–	–	–	–
ANTIGEN CONTROL	–	–	–	–	–	–	–	–	–	

Patte n A

MRS EG. ♀ 62 (Ha hinot)

ANTIGEN D LUT ONS ⟶

SE UM DILUTIONS ↓

1	2	4	8	16	32	64	128	256	5 2	1024	SERUM CONTROL
2	●	●	●	●	●	●	●	o	o	–	–
4	●	●	●	●	●	o	o	o	o	–	–
8	●	●	●	●	●	o	o	–	–	–	–
16	●	●	●	●	o	–	–	–	–	–	
32	●	●	●	●	o	–	–	–	–	–	–
64	●	●	●	●	o	–	–	–	–	–	–
128	●	●	●	●	o	o	–	–	–	–	–
256	●	●	o	o	o	–	–	–	–	–	–
5 2	o	o	o	●	●	o	–	–	–	–	–
1024	–	–	–	o	o	–	–	–	–	–	–
ANTIGEN CONTROL	–	–	–	–	–	–	–	–	–	–	

Patte n B

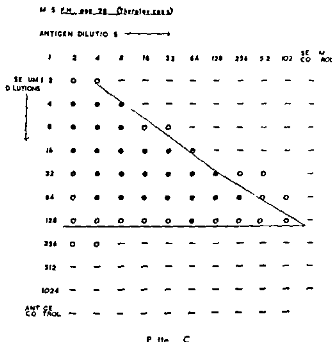


FIGURE 1 Thyroid complement fixation test in chessboard titration The antigen was a saline extract of a thyrotoxic gland (1 4 w/v) ● Complete inhibition of hemolysis ○ partial inhibition of hemolysis

antibodies A series of extracts from several glands were titrated against a positive serum of known titer preferably in chessboard and the most potent antigen was chosen for all the tests For screening purposes the sera were tested at dilutions of 1 5 and 1 20 after inactivation at 56° C for 30 minutes against antigen at a final dilution of 1 part thyroid tissue in o parts borate buffer For the test the serum antigen and 2 minimal hemolytic dilution (MHD) of complement (standard drops of each placed on opaque Perspex sheets with appropriate serum and antigen controls) were incubated for 1 hour in a humid chamber at 37° C before addition of the sensitized sheep cells The extent of hemolysis inhibition was read after a further 30 minutes at 37 C The results were expressed as + + + complete fixation at 1 20 dilution of serum + + complete fixation at 1 5 + partial fixation at 1 5 Sera which fixed complement at 1 o were tested by chessboard titration antigen and serum were made to react in serial dilutions up to a final antigen dilution of 1 4096 w/v and serum dilutions up to 1 10 4

The patterns obtained fell into several groups In the commonest pattern (Figure 1 pattern A) fixation occurred at high serum dilutions while

antigen could only be diluted to about 1 1 8 w/v In a few Hashimoto patients fixation also occurred with the highest antigen dilutions in the first few dilutions of serum so that a double rectangle resulted (Figure 1 pattern B) The third pattern (Figure 1 pattern C) showed a prozone and much stronger fixation was obtained as the serum was progressively diluted This phenomenon could be repeated with several different antigens and appears to be a property of the antiserum Patterns A and C were seen in thyrotoxicosis and subacute thyroiditis as well as in Hashimoto s disease and primary myxedema but so far the double rectangle pattern has been found only in Hashimoto patients with precipitins though this appearance could not be correlated with the titer of the precipitins Complement-fixing activity could still be demonstrated in sera kept at $-10°$ C for 1½ years

Coprecipitation with Radioactive Thyroglobulin

This method was evolved in order to study nonprecipitating thyroglobulin antibodies present in small amounts In principle the method involves the addition of radioactive antigen to the immune serum this forms a complex with antibody which can be precipitated by adding a rabbit antiserum to human gamma globulin capable of carrying down all the human thyroglobulin precipitins The radioactivity of the precipitate then gives a measure of the antibody content of the serum

The serum to be tested is incubated with either I^{131} thyroglobulin (purified from a thyroid taken at operation 36 hours after administration of 200 μc of I^{131}) or C^{14} thyroglobulin (obtained by synthesis of thyroglobulin in surviving human thyroid slices in the presence of a C^{14} algal protein hydrolysate as described by Roitt, Campbell and Doniach [33]) at 37° C for 1 hour An excess of the rabbit antiserum is added and after incubation at 37° C for 30 minutes and standing at 2° C overnight the precipitate is spun down thoroughly washed twice with cold saline and the radioactivity counted Knowing the specific activity of the antigen used a measure of the antibody level in the serum could be calculated by reference to a standard curve constructed with dilutions of a serum of known antibody content in normal serum The method is more sensitive at the lower levels of antibody where the proportion of antigen in the specific complexes is greater, but until it can be established that comparable antigen to antibody ratios are obtained with different sera in the region of extreme antigen excess the method can only be regarded as semiquantitative Levels of precipitating and nonprecipitating antibody down to 0 0 mg protein per milliliter of serum can be estimated

Incidence of Autoantibodies in Hashimoto's Disease and in Other Thyroid Disorders

Hashimoto's Disease

The sera of 69 patients untreated or treated with thyroid hormones were each studied by the Oudin tanned cell hemagglutination (TRBC) and complement fixation tests (CFT) the results are presented in Figure A The majority of the sera contained complement fixing antibodies of high titer and it is of interest that three of these were negative on the TRBC test One half of the sera tested had TRBC titers of 500 000 and all of these gave well defined precipitation zones in the Oudin test Significantly one of these sera was negative by CFT In sera with agglutination titers of 50 000 and 5 000 the majority gave positive Oudin tests but as the agglutination titers decreased the proportion of patients with positive precipitins fell off sharply

These results support the view that the hemagglutination test in general gives a measure of the level of thyroglobulin precipitins confirmation of this was provided by studies with the radioactive antigen coprecipitation method

A small number of Hashimoto sera behaved peculiarly in that the precipitin test with crude extract and purified thyroglobulin was positive despite a relatively low TRBC titer (25 to 250) In these cases the precipitin zone took from 7 to 10 days to develop and was of a diffuse appearance with indistinct boundaries It is conceivable that this represents reaction with another antigen present in small amounts in both extracts On the other hand Owen and McConahey have demonstrated the presence of a thyroglobulin like protein in the sera of some Hashimoto patients in these sera the thyroglobulin could form nonprecipitating complexes with circulating antibody and this would be expected to reduce the TRBC titer A precipitin could still result in the Oudin test by coprecipitation as antigen diffused increasingly towards these soluble complexes We have been able to show that incomplete antibodies do occur in the horse flocculation type of precipitin curve (see Figure 6) and that they are precipitated as more antigen is added

Following subtotal thyroidectomy the antibody levels declined in Hashimoto patients (Figure 2B) and in general were lower the greater the time which had elapsed since the operation The two sera which were negative on all tests were from patients operated upon 8 and 22 years previously Evidently thyroidectomy diminishes the antigenic stimulus for the autoimmune process Again a parallelism between the agglutination titer and the presence of thyroglobulin precipitins is suggested

35 30 25 20 15 10 5 0

NO OF PATIENTS

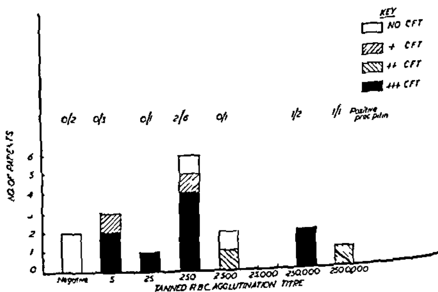
0/3 0/2 1/7 1/4 4/9 2/3 4/5 34/34 Positive precipitin

KEY
NO CFT
+ CFT
++ CFT
+++ CFT

Negative 5 25 250 2500 25000 250000 >2500000

TANNED RBC AGGLUTINATION TITRE

A Unoperated cases untreated or treated with thyroid hormones.



B Patients tested after subtotal thyroidectomy

Figure 2 Incidence of autoantibodies in Hashimoto's thyroiditis The tanned red cell agglutination titer is expressed as the reciprocal of the highest serum dilution given "one plus" agglutination The proportion of patients showing positive thyroglobulin precipitins in the Oudin test is shown above the appropriate column

Two sera with titers of 50 and 500 respectively failed to fix complement

The discordance between the agglutination and complement fixation test became more apparent still when similar studies were extended to other thyroid diseases The results presented in Table I clearly show that the two tests are providing a measure of different antigen antibody systems, and in fact it was demonstrated that the removal of precipitating

TABLE I THE INCIDENCE OF THYROID AUTOANTIBODIES IN VARIOUS THYROID DISORDERS

Disease	Total Number of Patients	Number of Patients without Antibodies	Number of Patients with Antibodies			
			+CFT +TRBC	+CFT −TRBC	−CFT +TRBC	+P
Hashimoto						
Unoperated	69	0 (—)	[illegible]	3	1	46
Postoperative	16	2 (12 5%)	12	0	2	4
Myxedema without goiter	49	17 (34 7%)	16	8	8	12
Thyrotoxicosis	102	27 (26 5%)	36	10	29	3
Nontoxic nodular goiter	102	58 (56 9%)	8	6	30	0
Subacute thyroiditis	18	6 (33 3%	11	0	1	7
Carcinoma of thyroid	18	13 (72 2%)	1	1	3	0

CFT complement fixation test TRBC tanned red blood cell agglutination P precipitin test in agar

antibody from Hashimoto sera by flocculation with thyroglobulin did not affect their complement fixation titer

Primary Myxedema

The incidence of high TRBC and complement fixation titers in sera from patients with myxedema lends further support to the view suggested by comparable histological features of the gland that this condition occurring in middle age and Hashimoto s thyroiditis have the same underlying disease process We find as do Goudie Anderson *et al*[13] that precipitins or high titer complement fixation are more often present in myxedema of short duration and that the tests tend to be negative in long standing cases due possibly to antigen exhaustion In two cases who had precipitins 1½ years ago the test has become negative in one and much weaker in the other during this interval Immunological tests

may prove useful in the diagnosis of early myxedema when the BMR and cholesterol values are still equivocal and I^{131} uptake is normal

Thyrotoxicosis

About 75 per cent of thyrotoxic patients have thyroid autoantibodies, though the titer is often low and only 2 per cent show precipitins Of our cases 45 per cent gave positive CFT, Goudie *et al* find these antibodies in 45 per cent of thyrotoxics using a slightly different method Positive TRBC tests were found in 64 per cent of our patients, and the most common titer was 250

Antibodies were found in untreated patients and in those treated for prolonged periods with antithyroid drugs The antibody reacted with the patient's own excised gland thus behaving as a true autoantibody Patients who were in prolonged remission after antithyroid drug treatment did not show an increased incidence of circulating antibodies and sometimes patients on these drugs and potentially still toxic had high titers in the CFT This suggests that autoimmunization is not a decisive factor in the 50 per cent of patients who are eventually cured after a course of drug treatment

It has been shown by previous workers[4] that thyrotoxic patients whose glands show patchy thyroiditis have an increased tendency to develop myxedema postoperatively and that this tendency increases with the extent of the lymphoid change We have found a strong correlation between circulating antibodies and patchy lymphoid change in thyrotoxicosis (9 of 11 cases) and Goudie *et al*[13] found the same in symptomless elderly women Several of our patients who had antibodies before operation and patchy lesions in their thyroid gland became hypothyroid after operation, but some patients with low antibody levels and a limited degree of lymphoid replacement have remained euthyroid for over a year following seven eighths removal of their thyroid It would appear that autoimmunity plays a role in the increased tendency to postoperative myxedema and we are now studying this aspect with a view to predicting this outcome before operation The relationship between preoperative serum antibody levels and the extent of lymphoid change assessed quantitatively by a statistical method[45] requires investigation Complete agreement between circulating antibody levels and histological findings cannot be anticipated since in the early stages of the autoimmune process antibodies formed locally might attach themselves to antigen and it is possible that serum antibodies would only appear when reticuloendothelial sites outside the thyroid became implicated

Postoperative myxedema is not due to a boosting effect on the antibody level by antigen released during the surgical procedure for we find that antibody titers are always lower after thyroidectomy The preoperative

antibody level and perhaps the type of antigen to which the patient is immunized may play a part in producing myxedema as well as the amount of gland removed

Nontoxic Nodular Goiter

This group of patients had a lower incidence of thyroid antibodies and the titer was usually low 37 per cent of cases gave positive tanned cell agglutination and the commonest titer was , Only 13 per cent had complement fixing antibodies and none showed precipitins

Subacute Thyroiditis

We have studied the sera of 18 patients who gave a definite history of an illness with fever and malaise and a painful thyroid swelling appearing fairly rapidly [45] Thyroid antibodies were found in 1 cases When the sera were tested 4 of these patients had progressed to a stage indistinguishable from Hashimoto s disease and had become myxedematous but had no thyroid enlargement Histological proof is not available in 5 of these cases and it may be argued that the acute thyroid swelling and pain in these patients may have been due to an inflammatory response connected with the autoimmune process as suggested by Paine *et al* [28] rather than to an underlying virus infection [1] One further patient became myxedematous within 2 months of partial thyroidectomy Of the 11 patients who recovered without sequelae 6 had no antibodies when tested In 3 patients who recovered the antibody titer rose for 3 months after the onset and regressed almost completely when the patient recovered [11]

Malignant Disease of the Thyroid

The differential diagnosis between carcinoma of the thyroid and Hashimoto s disease is particularly important since operation is essential in malignant disease and unnecessary or even harmful in chronic thyroiditis [7] Clinically the two conditions give rise to very firm goiters which are quite often indistinguishable on palpation In 18 cases of proved thyroid malignancy investigated immunologically 13 were negative by all methods 3 had weak tanned cell titers and one had positive complement fixation while both tests were positive in a case of reticulosarcoma of the thyroid coexisting with Hashimoto thyroiditis This type of case though very rare has received special attention in the literature [31] It is not known whether the malignant disease leads to autoimmunization or whether a sarcomatous change takes place in an already established chronic thyroiditis

Precipitins have not so far been obtained in a single case of carcinoma of the thyroid and this test may be the safest to rely on clinically in this connection since a positive result implies a very high level of antibodies

which we have only found in Hashimoto goiters and "prir myxedema

Nature of the Thyroid Antigens

The results obtained using the three methods described abov autoantibody detection indicate the complexity of the antigenic sy involved in thyroid autoimmunity and this is further borne out b independent behavior of the precipitating and complement fixing bodies in a given serum Our present views on the immune sy involved in thyroiditis are summarized in Table II

TABLE II Autoimmune Systems in Thyroiditis

Antigens	Antibodies	Source
Thyroglobulin	*Precipitins* three distinct lines in agar *Nonprecipitating* detected by red blood cell agglutination and coprecipitation with radioactive antigen	Colloid from a human thyrc
Complement fixing antigens	Chessboard titration suggests the presence of two distinct systems	Most abundant thyrotoxic gl

Precipitating Antigens

There is considerable evidence to support the view that thyroglob is the main antigen responsible for the precipitin reaction given by cr thyroid extracts with Hashimoto sera When set up in an Ouchterl plate against a serum containing precipitins the crude extract and thy globulin gave reactions of identity [8] Immunoelectrophoresis in agar g showed that the antigen in the crude extracts moved with the s mobility as did a purified thyroglobulin preparation [23] Further a Ha moto serum precipitated up to 90 per cent of the radioactivity c C^{14} thyroglobulin preparation labeled *in vitro* and up to 96 per cent the I^{131} of a thyroglobulin preparation isolated from the gland of a pat given I^{131} The heavy staining of the precipitin lines in agar plates the periodic acid Schiff technique [42] accords well with similar stain of the colloid in histological sections of the thyroid and with the fact t thyroglobulin is a mucoprotein having a carbohydrate content of per cent Although thyroglobulin preparations obtained from otl species have been shown to move as a single peak when studied boundary electrophoresis or by ultracentrifugation the nature of t 'salting out' curve observed with increasing concentrations of ammonu

sulfate or potassium phosphate led Derrien *et al* to suggest that thyroglobulin contained possibly three different components of a rather similar nature. It is of interest that when Hashimoto sera react in agar with human thyroglobulin up to three distinct precipitation zones can be seen with certain sera particularly when the reactants are not present in equivalent proportions (Figure 3). All the lines stain comparably with the periodic

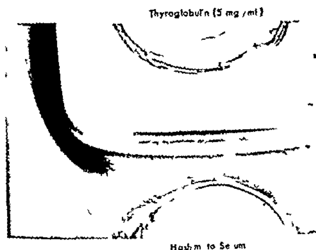


FIGURE 3 Ouchterlony plate showing multiple precipitin lines obtained with a Hashimoto serum against purified human thyroglobulin. The lines are stained with Azocarmine B

acid Schiff reagent and all contain iodine as evidenced by autoradiography (Figure 4). In certain cases the lines from two Hashimoto patients crossed each other establishing the nonidentity of the antigen fractions involved. Using a heterologous antibody Easty *et al* recently demonstrated the existence of three and possibly four antigens in human thyroglobulin. Thus it would appear that thyroglobulin is heterogeneous and contains at least three proteins with very similar properties but having independent antigenic identities.

The precipitating antibody is probably directed against normal rather than a modified thyroglobulin since identical results were obtained with extracts from normal postmortem glands and those prepared from thyrotoxic and nontoxic nodular thyroids obtained at operation. Also in the Coons fluorescent antibody technique the Hashimoto precipitin becomes localized in the colloid and in droplets at the apical margins of the follicular cells of a normal gland. Certainly purified thyroglobulin has been shown to be autoantigenic in rabbits and it would seem to be unnecessary to postulate the prior occurrence of a structural abnormality

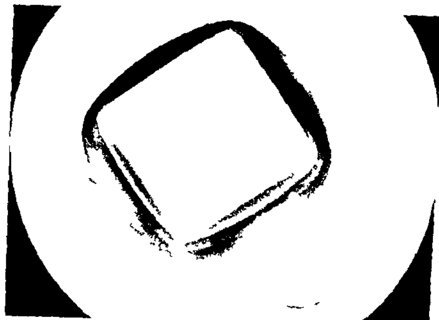


FIGURE 4 Autoradiograph of an Ouchterlony plate in which dilutions of a Hashimoto serum have reacted with I^{131} thyroglobulin (center well) purified from the gland of a thyrotoxic patient given 200 μc of I^{131} preoperatively Each of the three precipitin lines contains iodine

in human thyroglobulin to make it capable of stimulating autoantibody formation rather might the explanation for its antigenic activity be sought in a failure of the individual to acquire an immunologic tolerance to the protein owing to its normal imprisonment within the follicular structure of the gland

Complement fixing Antigens

Purified thyroglobulin only fixes complement with certain sera and then to a small degree Trotter Velyavin, and Wadhams[40] compared saline extracts of normal nontoxic goiter and thyrotoxic glands in complement fixation studies and found that an antigen reacting in this test was present exclusively in thyrotoxic glands They were able to sediment this antigen by centrifugation at 20 500 g for 1 hour leaving the precipitating antigen in the supernatant We have confirmed and extended these studies Fresh thyrotoxic gland was disrupted in 0 5 M sucrose in a Potter Elvehjem homogenizer and subjected to differential centrifugation The complement fixing antigen was localized in the microsome' fraction which sedimented at 105 000 g after removal of nuclear and mitochondrial fractions which were inactive Occasionally some activity could be demonstrated in the supernatant fraction[22] When a nontoxic nodular goiter and a normal gland were subjected to

the same procedure complement fixing activity against a Hashimoto serum was again recovered in the microsome fraction but was much weaker

The opportunity arose of studying the complement fixing activity of a single toxic adenoma which behaved like thyrotoxic tissue in that its raised iodine uptake could not be suppressed with triiodothyronine and of the normal opposite lobe from the same patient in which iodine uptake was absent While the adenoma extract gave positive fixation the normal lobe was inactive against a Hashimoto serum giving a chessboard pattern of type A (Figure 1) These results and the potent complement fixing activity of antigens obtained from thyrotoxic glands may suggest that this antigen is specific to thyrotoxicosis and that a similar antigen is only found in other thyroids when a toxic focus is present However patients with Hashimoto s disease myxedema and nontoxic nodular goiter as well as 16 per cent of elderly women with no overt thyroid disorders become immunized against this antigen and it would be necessary to postulate a very widespread incidence of toxic foci on this basis If on the other hand the antigen is present in normal functioning glands perhaps as a complex intracellular precursor of thyroglobulin its abundance in thyrotoxic glands might reflect a greater proportion of follicular cells and their increased secretory activity

When complement fixing activity is obtained with high antigen dilutions as in occasional Hashimoto sera giving chessboard pattern B (Figure 1) this activity can be demonstrated with purified thyroglobulin which may account for White s finding of complement fixation with such preparations [44] This type of serum also fixes complement with crude extracts of normal glands

Trotter and Belyavin have made a special study of this aspect and consider that this represents a separate antigen The antibody to this antigen occurs only rarely and is present in low titer We have obtained the double rectangle complement fixation pattern only in Hashimoto patients with precipitins Though this antigen appears to be present in preparations of thyroglobulin it may not be thyroglobulin itself

In a study of the complement fixation test with thyroid glands from several species sheep hog beef rat rabbit and guinea pig were negative but rhesus monkey and chimpanzee gave strong reactions with some Hashimoto sera This parallels our previous findings on the species specificity of the precipitin reaction

Characteristics of the Thyroglobulin Precipitin

The autoantibodies to human thyroglobulin are localized in the serum gamma-globulin fraction This was demonstrated both by precipitin reac

tions carried out on serum fractions eluted after zone electrophoresis on cellulose columns and by immunoelectrophoresis in an agar gel[33] Further the Hashimoto gamma globulins were indistinguishable from the normal in the Ouchterlony test when made to react with rabbit antisera prepared against either a Hashimoto serum or against human gamma globulin This accords with what is known of the antigenic similarity of specific and nonspecific gamma globulins

On the basis of the quantitative precipitin curves obtained with thyroglobulin the sera of Hashimoto patients could be classified into two main groups viz 'rabbit precipitin and horse flocculation' types[33] The first group exemplified by Figure 5 gave curves having the usual characteristics found with rabbit precipitating antibodies in that the antigen was completely precipitated in the region of antibody excess and was detectable by the ring test only in the supernatants at maximal precipitation and in antigen excess When I^{131} thyroglobulin was used as the antigen

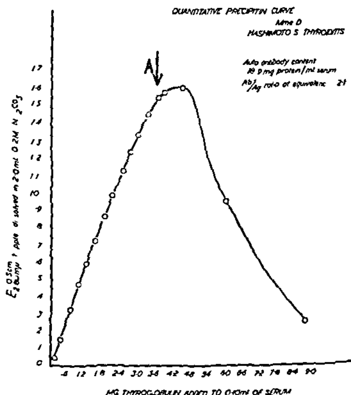


FIGURE 5 Quantitative precipitin curve of the "rabbit precipitin" type obtained with a Hashimoto serum At point A indicated by the arrow 95 per cent of added radioactive thyroglobulin was recovered in the precipitate Autoantibody content of the serum was 18.9 mg /ml Molar antibody antigen ratio at equivalence = 2 1

95 per cent of the isotope was recovered in the precipitate at the point A (Figure 5) no antibody was demonstrable in the supernatant by the ring test but one quarter of the remaining I^{131} was precipitated on addition of rabbit anti human gamma globulin serum thus revealing the presence of small amounts of soluble antigen antibody complexes It is perhaps surprising that a zone of equivalence of antibody and antigen can be attained in this system in view of the multiple nature of the antigens concerned but similar findings were obtained with many Hashimoto sera This would seem to be reflected in the single very narrow precipitation line frequently obtained in agar when the reactants are present in approximately equivalent proportions despite the appearance of multiple lines on dilution of the sera

The second group of which Figure 6 represents an example are characterized by the failure of the specific complexes to precipitate in the presence of excess antibody thus resembling horse flocculating

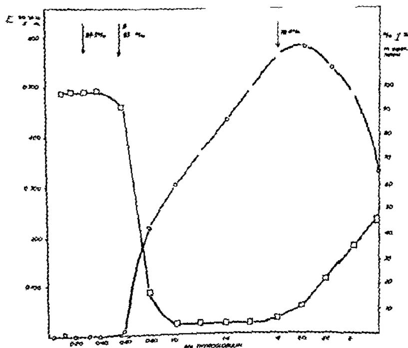


FIGURE 6 Quantitative precipitin curve of the horse flocculating type obtained with a Hashimoto serum and I^{131} thyroglobulin –O– optical density (E mμ) of the precipitate dissolved in 2.0 ml 0.1 M Na_2CO_3 –□– percentage of added I^{131} remaining in the supernatant At the arrowed points A B and C are given the percentages of supernatant radioactivity which could be coprecipitated by addition of a rabbit anti human gamma globulin serum indicating the presence of soluble complexes of thyroglobulin with the human antibody

systems In the region of antibody excess, substantially all the added I^{131} thyroglobulin remained in solution but addition of excess rabbit anti human gamma-globulin serum now brought down 90 to 93 per cent of the isotope demonstrating the presence of soluble complexes of thyroglobulin with the human antibody As was found with the serum used for Figure 5 almost all the antigen (96 per cent) was precipitated just before and at the point of maximal flocculation soluble complexes were again demonstrable in the supernatants from these precipitates

The molecular ratio of antibody to antigen in the specific precipitates was calculated on the basis of the following values for molecular weights thyroglobulin 650 000 and gamma globulin 170 000 (cf however Pressman *et al* [9]) The ratio varied generally from approximately 4 1 in the region of antibody excess to 1 at equivalence In contrast using the heterologous rabbit antibody to human thyroglobulin, Heidelberger [17] found ratios of up to 40 1 in the antibody excess zone

The levels of circulating thyroglobulin precipitins calculated from the quantitative curves frequently reach 5 mg of antibody protein per milliliter of serum and in the serum used for Figure 5 the strikingly high value of 18 9 mg of antibody per milliliter was obtained These figures represent a consistently high level of circulating antibody which probably results from a protracted continual leakage of antigen from the thyroid undergoing progressive destruction by the autoimmune reaction

Etiology of Chronic Thyroiditis

The results of immunological studies presented here and those published by other workers demonstrate the prevalence of thyroid autoimmunity in patients with thyroid disorders and its common occurrence in certain age groups in the absence of goiter The results also suggest that chronic thyroiditis and primary myxedema represent advanced examples of this process since these patients have by far the highest levels of circulating antibodies and the most extensive lymphoid replacement of the thyroid gland

It is probable that the patchy lymphoid infiltration found in the glands of 15 per cent of older women in the absence of overt thyroid disease [20, 34] is the mildest nonclinical manifestation of autoimmunity and that the immune process is initiated by localized release of thyroid proteins Goudie Anderson and Gray [14] found complement fixing antibodies in 16 per cent of women over 60 and were able to confirm the presence of patchy thyroiditis in the glands of some of these patients at postmortem The more extensive lesions were found in patients with the highest levels of circulating antibodies The incidence of localized thyroiditis is signifi

cantly higher in patients with goiter of any kind being 40 to 50 per cent in nontoxic nodular goiters and 80 per cent or more in thyrotoxic glands[16 21]. It is thus of considerable interest that we found circulating autoantibodies in 47 per cent of nontoxic goiter patients and in 75 per cent of thyrotoxics in some of these the glands were examined histologically and patchy lymphoid invasion was found. Thus it appears that the autoimmune process favors the already diseased gland. There is definite evidence of preceding thyrotoxicosis or subacute thyroiditis or of non toxic nodular goiter[35] in a minority of patients with Hashimoto s disease and it has been put forward by some authors that a much higher proportion of these patients have had previous subclinical hyperthyroidism. This has been denied by other investigators. Subclinical forms of various thyroid diseases may well precede chronic thyroiditis but this cannot be proved in the majority of cases.

Despite the presence of complement fixing antibodies in about o per cent of thyrotoxic patients in titers comparable to those found in Hashimoto s disease it is rare to observe the clinical progression from thyrotoxicosis to Hashimoto s disease or spontaneous myxedema we have only witnessed this change twice among several hundred thyrotoxic patients observed for to 5 years in the course of prolonged antithyroid drug treatment even though it is known that focal thyroiditis is more prevalent in long standing thyrotoxicosis. It appears therefore that the destructive chain reaction which leads to progressive lymphoid invasion of the thyroid gathers sufficient momentum to produce symptoms only in a minority of autoimmunized patients. Furthermore there is evidence that this process is not always irreversible. In subacute thyroiditis of virus origin massive breakdown of damaged follicles initiates the autoimmune process but when the infection subsides and the thyroid recovers antibody production ceases (Figure 7) and no progressive lesion results in the majority of cases. Thus the circulating antibodies may be a reflection of the degree of immunization without being the direct cytotoxic agent. This possibility is supported by our failure to produce thyroid lesions in a rhesus monkey by passive immunization with large amounts of Hashimoto serum which contained over 5 mg of precipitating antibody protein per milliliter as well as complement fixing activity. The Hashimoto serum cross reacted strongly with monkey thyroid in the precipitin test. The final concentration of precipitins in the monkey s circulation for several days following the injections was comparable to the level found in many Hashimoto patients yet none of the antibody was fixed by the thyroid *in vivo* as shown by Dr R G White using Coons s fluorescent antibody technique and no histological lesions resulted. A similar absence of direct cytotoxic action of the circulating autoantibodies was demonstrated by Professor Pulvertaft on tissue cultures of human thyroid gland[30] to which

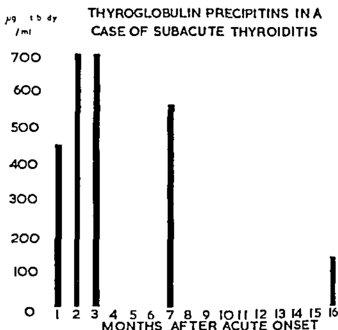


FIGURE 7 Quantitative thyroglobulin precipitin estimations in a case of sub acute thyroiditis proved by biopsy the precipitin levels were determined by the radioactive coprecipitation method

he applied several of our sera selected for their high antibody content, both of precipitating and complement fixing type

If as it appears probable the circulating antibodies are not in themselves responsible for the destructive lesions of thyroiditis one might postulate that these result from some form of immunological hyper sensitivity in the patients who destroy their own thyroid gland In the experimental thyroid autoimmunization produced in animals by Rose and Witebsky [35] the level of circulating antibodies did not always parallel the extent of thyroid destruction and it is possible that sensitization by the tubercle bacilli present in Freund adjuvant played a part in the cytotoxic effects

Thyroid autoimmunity may have interesting connections with other pathological phenomena of as yet unexplained etiology Luxton and Cooke [24] suggested a correlation between Hashimoto s disease and hepatic cirrhosis on clinical grounds We have since found precipitins in the sera of 3 patients who had both thyroiditis and liver disease and 3 further cirrhotic patients without overt thyroid disorders had small amounts of thyroglobulin antibodies as revealed by the tanned cell hemag glutination test Gajdusek and Mackay [12] [5] have recently reported the presence of complement fixing autoantibodies against liver and kidney

tigens in some cases of cirrhosis and the serum of a few of their patients io reacted with thyroid extracts It is possible that in cirrhosis the yroid autoimmunity is a secondary phenomenon due to cross reactions the antibodies to liver with thyroid antigens

Another interesting connection exists between the thyroid and the renal gland It has been noted for many years that extensive focal yroiditis is a frequent postmortem finding in patients who die of ddison s disease particularly when this is due to primary or so called oxic atrophy of the suprarenal [37, 43] Anderson *et al* [1] have recently ported the presence of autoantibodies against both adrenal and thyroid tracts in a patient who had a lymphadenoid goiter and Addison s sease and in another Addisonian patient without overt thyroid disease xperimentally Colover and Glynn [4] have obtained adrenal atrophy in uinea pigs immunized against homologous adrenal extracts Lastly axton [2, 23] has drawn attention to the frequent association of Paget s sease of bone with lymphadenoid goiter and of the occasional finding mild forms of nephrosis in patients with Hashimoto s disease Immuno gical studies may shed new light on these conditions as well as extend ur understanding of the antigenic relationships which exist between the llular constituents of different organs

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22

*Autoimmune Hemolytic Disease Some Experiences and Some Unsolved Problems**

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It is now fifty years since Chauffard and Widal and their respective colleagues first suggested that certain cases of hemolytic anemia might be due to an antibody like hemolysin or autohemagglutinin in the patient s serum Scattered bits of evidence to support this concept collected in the literature in the following years It was not until 1946 when Boorman Dodd and Loutit,[2] and later Loutit and Mollison[16] utilizing the then recently introduced antiglobulin reaction of Coombs Mourant and Race[4] demonstrated that the red cells of many of these patients were coated with an antibody like protein substance that the modern era of study of acquired hemolytic disease began Although these red cell coating substances have since been well characterized their basic nature and mechanism of production remain unknown It is probably fair to say that at present the consensus is that these substances are in all probability antibodies and that the designation autoimmune hemolytic disease (AHD) is thus justified

This paper will attempt to review briefly the clinical experience with these diseases encountered by the hematology group of the University of Rochester point out and illustrate some of the principal unsolved problems relating to AHD particularly those problems of an immunological nature and suggest a number of lines along which further investigation might proceed

Autoimmune hemolytic disease may be defined as a group of disorders characterized by the production by the patient of an antibody like substance which acts to shorten the life span of the patient s own red cells Idiopathic and symptomatic cases are recognized This definition is not entirely satisfactory for its application to any group of cases depends upon the criteria employed for recognition of the presence of red cell autoantibodies Many authors have included as AHD only cases with

* The observations reported in this paper were assisted by research grants from the U.S Public Health Service (A 537) and the Office of the Surgeon General Department of the Army (DA-49 MD-632)

positive direct antiglobulin tests of the red cells As will be pointed out there is increasingly strong evidence that the antiglobulin test does not detect red cell sensitization in all instances and that as more sensitive serological methods become available more cases will be included with confidence in the AHD group

A further difficulty in classifying cases of AHD relates to the diverse serological characteristics of the red cell autoantibodies[6, 7] At present we are unable to decide what relationships may exist between cases showing these diverse serological properties and which groups of cases may justifiably be considered together These questions are of obvious importance for understanding the biological process of autoimmunization as well as the problem of AHD

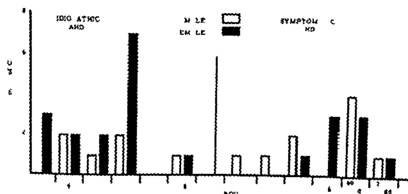
Table I summarizes briefly some of the main observations on cases of AHD having so called warm antibodies[18, 34, 3, 36] A second group of cases having frank hemolytic anemia of a chronic or recurrent type without demonstrable autoimmune antibodies is summarized in Table II Since 1944 we have encountered 38 adults and 4 children with a frank hemolytic state in whom clear cut evidence of the presence of an auto antibody could be demonstrated These patients have been followed for from 1 to 15 years and only those patients surviving into the period when the antiglobulin test was available (1946) are included The nature of the autoantibodies detected in these cases is also tabulated in Table I Several patients had moderate titers of serum cold agglutinin but in no instance did the primary red cell coating antibody show significantly greater activity at 4 C than at 37 C or at room temperature Thus in none of these cases was the antibody of the cold type described by Dacie and others The primary disorder in the cases of symptomatic AHD was in 15 instances a malignant disorder of the reticuloendothelial system and was found in about the same proportion as was encountered by other investigators[8, 10]

During this same period we have encountered 9 patients with reticulo-endothelial malignancies associated with overt hemolytic anemia but with no evidence of autosensitization as judged by repeatedly negative red cell antiglobulin tests and absence of serum panantibodies[27] These patients were otherwise entirely comparable, clinically and hematologically with the symptomatic cases in which antibodies could be demonstrated A number of similar patients with mild hemolytic processes have been encountered but are not included in this group The possible relationship of this group of patients to the symptomatic cases of AHD is of great interest

Figure 1 summarizes the principal features of a case from this latter group This 53 year old man has had chronic lymphocytic leukemia since mid 1955 and has had at least two attacks of overt hemolytic anemia

TABLE I SUMMARY OF CLINICAL EXPERIENCE WITH AUTOIMMUNE HEMOLYTIC DISEASE FOR THE PERIOD 1944–1958 47 CASES

	Idiopathic		Symptomatic	
	Males	Females	Males	Females
Ages ??–77 years	6	15	9	8
Ages 13 months 14 years	3	1	0	0
Number of Cases in Which Antibodies Were Detected				
Direct positive antiglobulin	9	14	9	8
Serum antibody	2	6	?	0
Cold antibody type	0	0	0	0
Diagnoses in Symptomatic Cases				
Reticulum cell sarcoma			0	4
Lymphosarcoma			?	0
Hodgkin s disease			0	1
Chronic lymphocytic leukemia			5	3
Sarcoidosis			1	0
Vasculitis			1	0
Present Status of Cases				
Dead	1	?	7	7
Alive with disease	4	4	?	1
Apparently well	4	10	0	0



Excessive transfusion requirements and shortened life span of Cr tagged red cells have documented the presence of the hemolytic state At all times his red cells have given negative direct antiglobulin reactions using reagents prepared by immunizing rabbits with whole human serum Serum antibodies have not been found Splenectomy terminated the hemolytic process the first time after steroid therapy had failed

TABLE II SUMMARY OF 9 CASES WITH FRANK HEMOLYTIC ANEMIA RETICULOENDOTHELIAL MALIGNANCY AND NEGATIVE RED CELL ANTIGLOBULIN TESTS

Age range	7 to 68 years
Sex	6 males 2 females
Diagnoses	Hodgkin s disease 4
	Chronic lymphocytic leukemia 2
	Lymphosarcoma 1
	Reticulum cell sarcoma 1
	Multiple myeloma 1

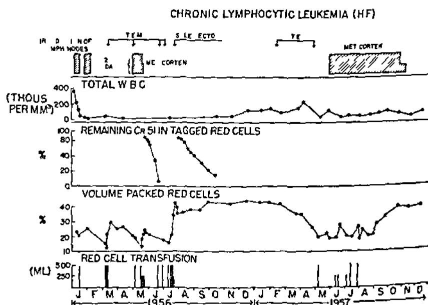


FIGURE 1 Clinical course of a 53 year-old man with chronic lymphocytic leukemia acquired hemolytic anemia and negative red cell antiglobulin test

Recently, Dr Richard Rosenfield of New York City has kindly made available to us an anti human protein rabbit serum he has prepared by immunizing the animals with a suspension of specific antigen antibody precipitate that has been exposed to fresh human serum and subsequently washed This antigen presumably contains human protein only as those fractions of complement absorbed to the preformed specific precipitate Utilizing this serum as in the antiglobulin test the red cells of this patient are strongly agglutinated The red cells of one other patient with chronic lymphocytic leukemia but without a demonstrable hemolytic state have also been found to react strongly with this serum Although the signifi

cance of these and similar observations is not yet certain they suggest that this serological method may be capable of detecting red cell protein coating possibly by a fraction of complement and mediated by auto antibodies in at least some of the cases of this group There is also accumulating evidence for the existence of red cell isoantibodies of an order of reactivity undetectable by currently available methods both in man and dogs manifest by their capacity to shorten the life span of apparently compatible transfused normal red cells

The mechanisms by which autoantibodies initiate the processes of red cell destruction have also been studied extensively in the last ten years[8, 13] Studies of the effects of isoantibodies and heteroantibodies upon red cells *in vivo* and *in vitro* have been of great use in understanding these mechanisms since many analogies exist between these two types of erythrocyte antibody interactions[4] Intravascular hemolysis with resultant hemoglobinemia sequestration of either spherocytic or auto agglutinated red cells erythrophagocytosis and possibly metabolic injury of the antibody coated red cells all seem to be modes of red cell destruction operating in AHD[23]

Some patients with AHD have evidence of red cell autosensitization only during periods of active hemolysis However many cases show persistently positive red cell antiglobulin tests even during periods of apparent remission of the hemolytic process[3] In such cases no apparent serological change accompanies reactivation of the hemolytic process This suggests that other serological or physiological components of the *in vivo* hemolytic system actually regulate the rate at which red cells are destroyed Elucidation of the nature of these factors would be a major step forward in understanding the processes by which antibodies injure cells

The association of idiopathic AHD with idiopathic thrombocytopenic purpura (ITP) is well known and Evans and associates[10] have suggested that these two disorders represent the extremes of a spectrum of manifestations of basically related processes In our series of adult idiopathic cases 2 patients have had frank attacks of ITP 3 others have had thrombocytopenia of moderate to marked degree during periods of active hemolysis and 7 patients have also shown significant degrees of leukopenia in association with active AHD Figure illustrates the course of a patient *a 31 year old man with fatal idiopathic AHD who had frank clinical* ITP at a time in the course of his illness when the hemolytic process was entirely quiescent Autopsy of this patient revealed only hyperplastic lymph nodes and did not establish the presence of an underlying disease state

Harrington and others[11] have shown the presence of antiplatelet substances in the plasma of certain patients with ITP These substances

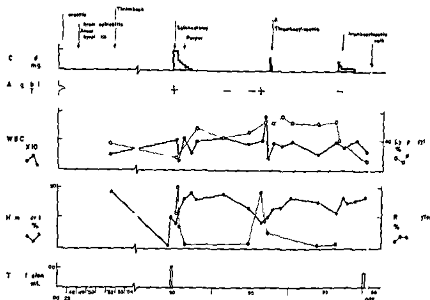


FIGURE 2 Clinical course of a 36 year old male with idiopathic AHD Note the attack of thrombocytopenic purpura in mid 1957 at a time when the hemolytic process was quiescent Two previous episodes occurred during or just after episodes of vigorous hemolysis

exhibit properties that suggest they may be antibodies The technical difficulties encountered in demonstrating these antiplatelet factors serologically have greatly hindered the elucidation of their basic nature Clarification of the relationships which may exist between AHD and thrombocytopenic and leukopenic states which may also have an autoimmune basis seems to be a matter of importance

AHD is not exclusively a disease of man We have observed two cases of AHD in dogs one a fatal idiopathic process occurring in a cocker spaniel[18] and the other symptomatic of lymphocytic leukemia in a mongrel In both instances the red cell antiglobulin test was strongly and persistently positive The clinical manifestations of the disease were virtually identical with the human counterpart This observation seems to be of importance in that many environmental factors not common to man and animals cannot be implicated as etiological factors in AHD

Efforts to produce red cell autoantibodies and AHD regularly in experimental animals have been unsuccessful Two groups of investigators have reported the development of transient positive red cell antiglobulin reactions in a few animals injected with their own blood modified in various ways[15, 9] However these animals did not show signs of hemolysis This is not unexpected in that some state of immune hyperresponsiveness may

be required for the induction of red cell autoimmunization The route of presentation of the antigen may be of less importance

The major problems to be faced in understanding the pathogenesis of AHD relate to the status of the red cell coating substances as antibodies demonstration of the antigenic red cell factors and the means by which these factors induce the production of antibodies The majority of the available evidence regarding the *antibody nature of the red cell reactive* substances is inferential in character These substances are proteins many apparently gamma globulins with many of the serological properties of known isoantibodies Many patients with AHD are easily immunized with foreign red cell antigens to which they are exposed by transfusions [1] They may also exhibit a number of nonspecific serological phenomena such as positive Wassermann reactions and rises of heterophil anti bodies [7, 25] These manifestations may also be related to facile production of a whole range of antibody substances by the patient s immune apparatus

The most convincing point supporting the view that these proteins are antibodies is that at times they exhibit specificity for various human blood group factors present in the erythrocytes of the patient himself In effect this may be interpreted as an identification of the responsible antigen in these cases In most instances these blood group specific autoantibodies have been directed against one or more antigens of the Rh complex Cases having antibodies specific for the red cell factors e c C D and Jk have been reported [8, 3, 31] At times these autospecific components were accom panied by nonspecific or panantibodies of the usual type

Recently we have had the opportunity to study 10 red cell eluates from 6 patients utilizing the red cells of a person with suppressed red cell repre sentation of the H antigen the rare so called Bombay type designated OcOc [14] However all of these eluates reacted with these cells suggesting that none of the eluates have specificity for any red cell factor influenced by this supressor gene The antigen responsible for autoimmunization in the average case has not been identified thus far It may not have specificity but may be a factor normally present in all human red cells It should be noted that combinations of two or more specific auto antibodies recognizing antigens with high incidences in the population at large might appear to be panantibodies The serological problem *inherent in studying this possibility is exceptionally formidable*

The well known phenomenon of virus hemagglutination has raised the possibility that a similar process may occur *in vivo* in AHD patients [3, 1] Alternatively it may be suggested that virus adsorption onto the red cells followed by attachment of antiviral antibodies might be the mechanism by which the erythrocyte protein coating is produced There is little direct evidence to support either of these theories and indeed substantial evidence and theoretical considerations against them [7] However the

occurrence of acute AHD following known and suspected viral infec tions still suggests the possibility of a more indirect relationship Red cells might be altered by virus action in such a way that they become effective antigens Viruses or for that matter any number of heterogenic antigens could conceivably produce antibody responses which cross react with some component of the red cell but again direct evidence is lacking and this possibility also seems theoretically farfetched The role of infectious processes in general may be related only to the alteration produced in the patient s reticuloendothelial lymphatic apparatus and antibody producing mechanisms The common factor may be induction of a state of enhanced immune responsiveness possibly for a limited range of antigens

A possibility deserving more serious consideration is that somehow possibly by a number of independent mechanisms the red cell surface may become altered in its physiochemical structure, with the result that it then nonspecifically adsorbs certain normal plasma proteins such as comple ment Again this mechanism is undemonstrated experimentally hard to accept theoretically and difficult to reconcile with the known specificity of at least some autoantibodies

Demonstrable red cell autosensitization apparently resulting from hap ten action of the drug Faudin has been reported by Harris and colleagues and several other drugs have also been incriminated [1, 23] This mechanism resembles that which has been shown to occur in certain cases of drug induced thrombocytopenia and leukopenia [1, 0] but it probably does not for many cases of AHD

Strong support has been gained for the concept that AHD is a disorder characterized primarily by abnormal protein production and that the production of proteins that act as specific or nonspecific red cell autoanti bodies is only another manifestation of a basically disordered pattern of protein synthesis [25] This concept would involve a remarkable but con ceivable functional alteration in protein synthesizing cells We must not lose sight of the possibility that what we now regard as autoimmunization may occur by a number of basically different mechanisms

I should like to conclude with an attempted *formulation* of ways in which some of the currently emerging biological principles might help us in construction of a theory of autoimmunization Of key importance seem to be the recent demonstrations of chronic thyroiditis as an autoimmune disorder [2, 24] Although the route by which the antigen thyroglobulin, gains access to the antibody producing mechanism in the spontaneous cases occurring in man has not yet been demonstrated this seems to be a matter of secondary importance These observations seem to me to show that autoimmunization is at least biologically possible As a corollary Ehrlich s long held view of horror autotoxicus is shown to be a limited

principle not applying to all substances within the body of the organism even though they be structurally and functionally part of the organism. Rather it might be proposed there are at least two kinds of "self" — that which the immune mechanism recognizes as self by virtue of previous or frequent contact and those substances which this apparatus has not previously encountered because they are anatomically and functionally excluded from antibody producing cells under normal physiological circumstances. When this latter type of "self" substance is exposed to the immunological apparatus it may evoke an immune response depending upon the antigenicity of the substance and the degree of responsiveness of the organism at that time.

The problem presented by AHD then would have two main aspects. How do the red cell antigens gain access to those cells capable of producing antibodies and what factors then regulate the antibody response that will be evoked? Regarding the first one could ask: Are there red cell factors which are structurally so located that they are functionally inaccessible as antigens? Are there red cell antigens of this type, even those having group specificity, which develop late enough in fetal life to be regarded immunologically by the organism as "non self"? Could these antigens then become available as a result of several kinds of alterations in the cell? It is noteworthy that the development of isoantibodies to the so called "minor" red cell antigens seems to be a rare event following transfusions even though we now feel that almost every transfusion is potentially antigenic. Is this explained by the current assumption that these factors are "weak" antigens or are they just unavailable?

Regarding the antibody response evoked by exposure to a red cell antigen one might assume that AHD patients have enhanced responsiveness at least for certain classes of antigens. This might be the result of disease processes and be either permanent or temporary depending upon the nature of the underlying disorder. Or in idiopathic cases of AHD the enhanced responsiveness could conceivably be of a statistical nature. The biological property of antibody response is probably distributed over a wide normal range like most other characteristics. Are patients with idiopathic AHD those who fall at the extremely responsive end of the spectrum of reactivity to this class of antigens?

Thus to initiate the process of red cell autoimmunization two events may be required: some sort of permanent or temporary alteration of the red cell antigens and the occurrence of this alteration at a time when the organism recognizes this antigenic stimulus and is capable of responding strongly to it. It is unfortunate that we do not have data to support or for that matter disprove this concept.

In closing I would direct your attention to the criteria which Witebsky and colleagues[22] have recently proposed should be fulfilled in order to

prove the role of an autoantibody in the pathogenesis of any disease state These criteria which may become the equivalent of Koch s postulates in the field of autoimmune disorders are (1) the direct demonstration of free circulating antibodies that are active at body temperatures or of cell bound antibodies by indirect means ( ) the recognition of the specific antigen against which this antibody is directed (3) the production of antibodies against the same antigen in experimental animals and (4) the appearance of pathological changes in the corresponding tissues of an actively sensitized experimental animal that are basically similar to those in the human disease We are obviously a long way from fulfilling these criteria for AHD but I believe we can already look forward to a day when they will be satisfied for this challenging and intriguing group of disorders

ACKNOWLEDGMENTS

In preparation of his paper the author has drawn freely upon many of the clinical and laboratory observations made by present and past colleagues who have been members of the hematology group of the University of Rochester School of Medicine and Dentistry and its Department of Medicine He is especially indebted to Drs L F Young Gerald Miller S B Troup and Heinz Valtin and to Miss Norma Trabold Detailed reports of the clinical experience cited in this paper are in preparation

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23

Antinuclear "Antibodies" in Lupus Erythematosus

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Systemic lupus erythematosus is one of the diseases of connective tissue thought to be a member of the rheumatic disease family. Many organ systems of the body may be involved, including skin, blood forming tissues, kidney, joints and serous surfaces. One of the features of this disease is the appearance of many substances in the serum which react with constituents of various cells or of the serum itself. Thus patients with systemic lupus erythematosus may have a hemolytic anemia with positive Coombs tests, a leukopenia sometimes with accompanying leukoagglutinins, a thrombocytopenic purpura, a false positive serologic test for syphilis, a circulating gamma globulin which interferes with clotting, and kidney lesions in which there is deposition of gamma globulin in the glomerulus. The hallmark of this disease, however, is the lupus erythematosus cell.

The L.E. cell was first described by Hargraves. The formation of this cell is thought to proceed as follows: serum from certain patients with this disease reacts with white cells *in vitro* causing a swelling of the nucleus of the white cell. The swollen nucleus is then extruded into the surrounding medium and is phagocytized by another polymorphonuclear leukocyte. This latter leukocyte containing the swollen nucleus as an inclusion body is called the L.E. cell. The factor which causes this change has been shown by Haserick to be a gamma globulin which he thought to be an abnormal gamma globulin.

The morphologic appearance of L.E. cells strongly suggests that a reaction occurs which directly involves the cell nucleus. Miescher[5] was the first to give direct evidence for such a reaction when he demonstrated that lupus serum could be freed of its ability to induce L.E. cell formation by absorption with isolated cell nuclei. Recently various groups of workers including our own[4,6,7] have studied the nature of this reaction. The present report is a summary of the evidence from our work which leads us to believe that the factor inducing this cell change is a member of a family of substances, probably antibodies, which appear in the serum of

patients with lupus erythematosus and which are directed toward different constituents of the nucleus of the patient's cells.

Table I demonstrates the reaction between cell nuclei and the L.E. cell factor. It may be seen that after the absorption of serum with isolated

TABLE I. ABSORPTION OF L.E. CELL FACTOR BY NUCLEI AND NUCLEOPROTEIN

Type of Nuclei	Treatment of Nuclei	L.E. Test			L.E. Cells Formed When Nuclei Incubated with WBC	
		Serum *Before* Absorption	Serum *After* Absorption	56° Eluate	Nuclei Exposed to L.E. Serum	Nuclei After 56° Elution
Calf thymocytes		++++	–	++	++++	
Rabbit PMN	None	++++	–	+	++++	+++
Human monocyte		++++	–	+	++++	+++
Rabbit PMN	Prior exposure to normal serum	++++	–	+	++++	
Isolated nucleoprotein	None	++++	–	±	+++	

nuclei from various organs and species the serum is no longer capable of inducing L.E. cell formation. If these nuclei are washed with cold saline after absorption and are then eluted in saline at 56° C. for a few minutes a material is recovered in the supernate which is capable of inducing L.E. cell formation. It may also be seen that nuclei which were exposed to lupus serum and then washed are readily phagocytized to form L.E. cells when incubated with fresh white cells. After elution of such nuclei the nuclei are still phagocytized though to a less intense degree indicating an incomplete elution of the factor. Nuclei exposed to normal serum or various other sera with hypergammaglobulinemias are not so phagocytized. It may also be seen that exposure of the nuclei to normal serum does not interfere with their subsequent ability to absorb the L.E. cell factor. This suggests that there are specific binding sites on the nucleus with which the L.E. cell factor reacts that are not blocked by normal serum globulin. Though the data are not presented in this table it is possible to alter the affinity of the nucleus for the L.E. cell factor by treating the nucleus with deoxyribonuclease. Thus if one treats the nuclei with DNase sufficient to remove only part of the DNA, then the ability of these nuclei to absorb the L.E. cell factor is reduced. If, however, one treats them with sufficient DNase to remove all of the DNA, then such nuclei cannot subsequently

absorb the L E cell factor On the other hand treatment of the nuclei with ribonuclease in no way alters their affinity for the factor It has also been possible to reduce or prevent the absorption of the factor on nuclei by prior treatment of the nuclei with protamine a basic protein thought to bind the phosphate groups of the DNA A similar result is obtained by treatment of the nuclei with atabrine a substance with therapeutic effect in this disease which is basic in nature and is known to form strong bonds with deoxyribonucleic acid and deoxyribonuclear proteins

It is not necessary however to have whole nuclei in order to absorb the L E cell factor As can also be seen from Table I isolated nuclear nucleoprotein obtained from a one molar sodium chloride extract of nuclei and precipitated is capable of absorbing this factor just as effectively as was the whole nucleus This nucleoprotein after absorption of the factor and many subsequent washes will also be phagocytized to form cells very similar to L E cells when incubated with normal white cells The absorption of L E cell factor on nucleoprotein is demonstrated not only by the disappearance of the factor from the serum and the phagocytosis of the washed nucleoprotein by white cells but also by an absolute increase in the amount of protein on the nucleoprotein after the absorption has taken place This increase in protein of the nucleoprotein is accompanied by an observable swelling of the nucleoprotein The adherence of gamma globulin to the nucleoprotein can also be demonstrated by the fluorescent antibody technique Thus it would seem that there is a direct affinity between a circulating material in the serum of lupus patients and the whole cell nucleus or the nucleoprotein constituent of the nucleus

The nature of the factor in the serum can be explored by examining the 56 C eluate from nuclei which have absorbed the factor This eluate which is capable of inducing L E cell formation contains protein which is entirely gamma globulin and is capable of cross reacting completely with antiserum to normal gamma globulin This suggests that the factor in the serum is not different immunologically from the other gamma globulin of normal human beings This evidence plus the identification of the L E cell factor in the gamma globulin component of serum on electrophoresis and the demonstration that the factor sediments in an ultracentrifugal field with an S rate of approximately 7 similar to that of the great bulk of normal human gamma globulin indicates that the factor in all probability is not an abnormal protein but is similar to the spectrum of antibody proteins of the human being

At first it appeared that patients with lupus possessed one serum factor capable of reacting with the cell nucleus However when the complement fixation technique was used to study the reaction between lupus serum and cell nuclei evidence appeared suggesting the presence of a number of different factors capable of reacting with cell nuclei These

complement fixation studies were carried out by the standard serum dilution technique using two 100 per cent units of guinea pig complement. End points were read visually as degrees of hemolysis and appropriate controls of both serum and antigen were included.

In Table II it may be seen that various lupus sera with different capacities to induce L.E. cell formation fixed complement with isolated

TABLE II. Complement Fixation with Calf Thymus Nuclei

Serum	L.E. Cell Formation	Serum Dilution 2	4	8	16	32	64	128	256	512
Syst lupus eryth (Ter)	Strong	4	4	4	4	4	4	4	4	4
Syst lupus eryth (Ca)	Strong	4	4	4	4	4	4	4	4	2
Syst lupus eryth (Fr)	Strong	4	4	4	4	4	4	4	3	0
Syst lupus eryth (Ne)	Strong	4	4	4	4	4	4	0	0	0
Syst lupus eryth (St)	Strong	4	4	4	4	3	0	0	0	0
Syst lupus eryth (Ca)	Medium	4	4	4	4	0	0	0	0	0
Syst lupus eryth (Hi)	Medium	4	4	4	1	0	0	0	0	0
Syst lupus eryth (Ro)	Medium	4	4	4	2	0	0	0	0	0
Syst lupus eryth (Sa)	Weak	4	1	0	0	0	0	0	0	0
Syst lupus eryth (Ma)	Weak	0	0	0	0	0	0	0	0	0
Syst lupus eryth (Ya)	Weak	0	0	0	0	0	0	0	0	0
Misc hyperglobulinemias	(9)	0	0	0	0	0	0	0	0	0
Rheumatoid arthritis	(21)	0	0	0	0	0	0	0	0	0
Misc diseases	(16)	0	0	0	0	0	0	0	0	0
Normal sera	(15)	0	0	0	0	0	0	0	0	0

cell nuclei. The titers varied and occasionally sera did not fix complement. It will also be seen that various sera from other diseases employed as controls did not show such a complement fixation. Table III demonstrates that when various constituents of the nucleus were used as antigens in the complement fixation system, different patients possessed different capabilities of reacting with the various constituents. For example one strong L.E. cell forming serum fixed complement with nuclei, nucleoprotein, and DNA but not with histone. Another equally strong L.E. cell forming serum fixed complement with nuclei, nucleoprotein and histone but not with DNA. It appears therefore that patients develop groups of serum factors which react with different nuclear constituents and that the character of these groups may differ from patient to patient.

Table IV demonstrates again the differing nature of these complement-fixing factors. The figures given under the columns of absorbed and unabsorbed serum represent the highest serum dilution at which complement was fixed. It will be seen with patient Fr that absorption of the serum with nuclei or nucleoprotein removed the complement fixation factors for many of the different nuclear constituents and reduced or removed L.E. cell inducing ability. However the absorption of the serum

TABLE III Complement Fixation by Representative L E Sera with Various Nuclear Constituents

Serum	L E Cell Formation	Antigen Used in C F	2	4	8	16	32	64	128	256	512	1024
							Serum Dilution					
Tr	Strong	Nuclei	4	4	4	4	4	4	4	4	2	0
		Nucleoprotein	4	4	4	4	4	4	4	4	2	0
		DNA	4	4	4	3	0	0	0	0	0	0
		Histone	0	0	0	0	0	0	0	0	0	0
Fr	Strong	Nuclei	4	4	4	4	4	4	1	0	0	0
		Nucleoprotein	4	4	4	4	4	4	4	2	0	0
		DNA	0	0	0	0	0	0	0	0	0	0
		Histone	4	4	4	4	0	0	0	0	0	0
Me	Strong	Nuclei	4	4	4	0	0	0	0	0	0	0
		Nucleoprotein	4	4	4	4	4	4	0	0	0	0
		DNA	4	4	4	4	2	0	0	0	0	0
		Histone	0	0	0	0	0	0	0	0	0	0

TABLE IV Effect of Absorption of Serum with Nuclear Constituents upon Complement Fixation Titers and L E Cell Formation

Serum	C F Antigen	Unabsorbed Serum	Serum Absorbed with Nuclei	Serum Absorbed with Nucleoprotein	Serum Absorbed with DNA
Fr	Nuclei	64	0	16	64
	Nucleoprotein	256	0	16	
	DNA	0			
	Histone	8	0	0	
	L E prep	++++	Negative	Weak	++++
Me	Nuclei	8		0	
	Nucleoprotein	64		0	
	DNA	32		0	
	Histone	0			
	L E prep	++++		Negative	
Ne	Nuclei	64	8		64
	DNA	64	64		2
	L E prep	++++	Negative		++++

with DNA did not interfere with the complement fixation titer toward nuclei nor with the L E cell inducing capacity With patient Ne absorption of the serum with nuclei reduced the complement fixation titer toward nuclei and rendered the L E cell preparations negative but did not interfere with the DNA fixation titer On the other hand absorption

of the serum with DNA removed the titer against that antigen but did not interfere with the titer against nuclei or with the ability to induce L.E. cell formation. In this latter case, it would appear that the factor which fixes complement with DNA is not identical with the L.E. cell factor.

In view of the fact that nucleoprotein was capable of absorbing the L.E. cell factor, experiments were undertaken to determine whether the complement fixing factor for nucleoprotein was identical with the L.E. cell factor. These data are shown in Table V. The experiments were

TABLE V. EFFECT OF ENZYME TREATMENT UPON ABILITY OF NUCLEOPROTEIN TO FIX COMPLEMENT AND ABSORB THE L.E. CELL FACTOR

Pt	Antigen	Treatment	Serum Dilution 4	8	16	32	64	128	256	512	Absorption of L.E. Cell Factor by NP
Men	*NP	Control	4	4	4	4	4	0	0	0	++++
		DNase	2	0	0	0	0	0	0	0	0
		RNase	4	4	4	4	3	0	0	0	++++
		Trypsin	4	4	4	4	2	0	0	0	
	DNA	None	4	4	4	4	3	0	0	0	
Fr	NP	Control	4	4	4	4	4	4	4	0	++++
		DNase	4	4	4	4	4	4	0	0	0
		RNase	4	4	4	4	4	4	3	0	++++
		Trypsin	1	0	0	0	0	0	0	0	
	DNA	None	0	0	0	0	0	0	0	0	
Meh	NP	Control	2	0	0	0	0	0	0	0	+++
		DNase	0	0	0	0	0	0	0	0	+
		RNase	2	0	0	0	0	0	0	0	+++
		Trypsin	4	4	4	4	0	0	0	0	
	DNA	None	4	4	4	4	0	0	0	0	

*Nucleoprotein

conducted by treating the nucleoprotein with DNase, RNase and trypsin, examining the complement fixation titers obtained with these altered nucleoproteins and comparing them with the titers against unaltered nucleoprotein and DNA.

It will be seen that with patient Men the complement fixation titer against nucleoprotein was roughly equivalent to that against isolated DNA. The treatment of the nucleoprotein with DNase completely abolished complement fixation but treatment with RNase or trypsin had no effect. With patient Fr, however, who is an equally strong L.E. cell former and who did not fix complement with DNA, the treatment of the nucleoprotein with DNase and RNase did not alter the titer of fixation

but treatment with trypsin completely abolished this titer This suggests that in the first instance the serum was reacting with the nucleic acid portion of the nucleoprotein and in the second instance the complement fixation titer against nucleoprotein represented a reaction between the serum and the protein portion of the nucleoprotein

A third pattern emerged with patient Meh whose serum had no appreciable titer against nucleoprotein until that nucleoprotein was treated with trypsin At that point there appeared a complement fixation titer which was equivalent to the titer of this serum against isolated DNA This suggests that the protein of the nucleoprotein was masking the combining sites on the DNA of the extracted nucleoprotein but when protein was removed from combination with DNA DNA binding sites were freed which were then capable of uniting with the serum complement fixing factor Thus the nucleoprotein which at first appeared to be a uniform antigen subsequently was found to have various combining sites and the complement fixation factors of different sera were directed against different sites or combinations of sites

In the last column of this table it will be seen that treatment of the nucleoprotein with DNase prevented absorption of the L E factor from each of the three sera regardless of whether or not such treatment interfered with complement fixation between the serum and the nucleoprotein This indicates that the L E cell factor is separate from the complement fixation factors which react with nucleoprotein An intact DNA portion of the nucleoprotein appears to be necessary for the absorption of the L E cell factor on this nuclear constituent These data suggest that different patients possess groups of factors which fix complement with nuclear constituents At the moment it appears that the L E cell inducing factor is not identical with any of the complement fixing factors Rather it is another factor which has an affinity for nucleoprotein and in all instances studied, requires an intact DNA portion of the nucleoprotein

The interaction of L E cell factor on complement fixing factors with nuclei and nucleoprotein was readily demonstrated by absorption of serum with these insoluble materials Proof of a direct reaction between serum components and DNA was more difficult though this was suggested by the fixation of complement However recently it has been possible to obtain precipitin bands between DNA and sera of certain lupus patients utilizing a modified agar diffusion technique It has also been possible to obtain a precipitin like curve from the reaction between DNA and lupus serum in which an apparent antigen excess phenomenon was observed In these cases the reaction was conducted at an alkaline pH at which both the nucleic acid and the serum proteins would have negative charges and therefore nonspecific electrostatic combinations would be minimized

Most immunologic reactions between serum constituents and constituents of tissues have been demonstrated to possess either a species or an organ specificity. Neither of these conditions holds with the lupus serum factors. In Table VI it will be seen that the serum from lupus patients is

TABLE VI. COMPLEMENT FIXATION TITERS WITH NUCLEI AND DNA FROM DIFFERENT SOURCES

Serum	Antigen	Serum Dilution						
		2	4	8	16	32	64	128
Ga	Human monocyte nuclei	4	4	4	4	3	0	0
	Calf thymus nuclei	4	4	4	4	1	0	0
	Rabbit PMN nuclei	4	4	3	1	0	0	0
Mc	Human PMN DNA (leukemia)		4	4	4	4	0	0
	Human PMN DNA (empyema)		4	4	4	4	0	0
	Human lymphocyte DNA (leukemia)		4	4	4	4	3	0
	Calf thymus DNA		4	4	4	4	0	0
	Rabbit spleen DNA		4	4	4	4	0	0
	Salmon sperm DNA		4	4	4	4	4	4

capable of reacting with nuclei from different animals and different organs and it is capable of fixing complement with DNA obtained from different species and different organs within these species. Thus it would appear that the nuclear reacting constituents of lupus serum do not possess absolute organ or species specificity, though there may be some gradation of reactivity between species especially in the reaction with whole nuclei.

Thus in summary, in the serum of many patients with systemic lupus erythematosus there appear various factors, one of which is definitely gamma globulin in character, which can react with different constituents of the cell nucleus. One such factor causes specific morphologic changes in the nucleus. Other factors fix complement with different nuclear constituents. These might be considered to be a family of different antibodies directed toward the nuclei. If they are antibodies they are true autoantibodies, for not only does the L.E. cell phenomenon occur with the white cells from the patient from whom the serum is withdrawn but cell constituents from that same patient will react with the serum from that patient in complement fixation studies. Thus far all the evidence obtained in characterizing the serum factors suggests that they are gamma globulin proteins.

Final proof of an immunologic reaction, however, awaits reproduction of these phenomena in experimental animals. It is possible in rabbits to obtain factors which fix complement when reacted with nuclear constituents after immunizing these animals with whole nuclei and nucleoprotein. However, the sera of these animals have not developed the characteristic

pattern of a lupus serum they have not possessed the capacity to react with the nuclei of the rabbit's own cells nor has a single animal been found possessing a factor which reacts with the nucleic acid portion of the nucleus All of the reactivities thus far obtained appear to be directed toward protein constituents of the nucleus Therefore while no evidence has yet appeared contradicting the conclusion that the lupus serum factors are antibodies to the cell nucleus the supporting evidence is incomplete

It would be tempting to speculate that the appearance of a family of serum antibodies capable of reacting with nuclear constituents of most if not all tissues of the patient would have some relationship to the diffuse pathologic changes which occur in systemic lupus erythematosus However it must be said that there is no evidence to date which permits such a conclusion There is indeed no evidence that the lupus factors can gain access to the nucleus of a viable cell *in vivo* Nevertheless whatever the ultimate resolution of the problem of pathogenesis may be systemic lupus erythematosus is a disease characterized by the appearance of many different apparent antibodies to constituents of the patient's tissues and among these are a group of antibodies directed toward constituents of the cell nucleus This is the first time such a reaction involving the nucleus has appeared in a disease and certainly exploration of the mechanism which gives rise to such substances may prove a fruitful avenue of approach toward the ultimate understanding of the pathogenesis of the disease

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24

*Study of L.E. Cell Formation by Phase Contrast Microcinematography**

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We have studied suspensions of living human leukocytes treated with the serum of patients with acute disseminated lupus erythematosus by the technique of time lapse phase contrast microcinematography. In a preliminary note[4] we reported that the nuclear lysis observed in certain cells was accompanied by a specific opsonization of the nucleus. This was indicated by a phagocytosis of the lysed nucleus itself in the absence of any phagocytosis of cytoplasmic fragments. The result of this process was the formation of the L.E. cell. We thus obtained a direct morphological proof of the strictly antinuclear character of the factor present in the serum of these patients. We have continued our studies and verified and completed our earlier observations with the study of numerous additional serums. We will present in this paper an analysis picture by picture of the events which we have observed in our microcinematographic records. Our conclusions will be discussed and compared with the results of other authors. We will attempt with the aid of our recent experimental findings in collaboration with Seligmann[3] to demonstrate the nature of the serological principle responsible for the L.E. phenomenon. In a later paper we will draw certain conclusions from this work of a more general physiopathologic significance.

EXPERIMENTAL METHODS

Serums

The pathologic serums studied all came from patients with acute disseminated lupus erythematosus, in most instances in acute exacerbation, less frequently in remission. All produced typical L.E. cells in smears prepared by the technique of Zimmer and Hargraves.[37] Some of the

* This study was conducted at the Centre d'Immuno-Pathologie de l'Association Claude Bernard, Hôpital Saint Antoine, Paris. Professor Raoul Kourilsky is Director of the Center and the study was made in collaboration with J. Pinet, J. Terrassier and C. Develey.

patients were under treatment with cortisone The serums were employed either fresh or after preservation at $-30°$ C for up to 10 months They were used undiluted or in a dilution of 1 2 in buffered physiological saline or in Hanks balanced salt solution

Leukocytes

Human white cells were isolated from peripheral blood by the technique which we described earlier[46] They came most frequently from individuals with an increased sedimentation rate and a slight leukocytosis (between 10 000 and 15 000 white cells per cubic millimeter) less frequently from normal individuals and sometimes from patients with chronic lymphoid leukemia Leukocytes from compatible as well as incompatible donors were employed The blood (20 ml) is taken by venipuncture with crystallized heparin the final concentration of heparin being 1 10 000 If the sedimentation rate is increased the blood is used simply heparinized If the sedimentation rate of the red cells is normal we add in order to accelerate sedimentation a solution of polyvinylpyrrolidone (3 5 per cent in physiologic saline) in the proportion of 3 parts of blood to 2 parts of the solution The blood with or without the accelerator solution is then allowed to sediment for 30 to 45 minutes in an incubator at $37°$ C in a test tube 16 mm in diameter at an angle of 45 degrees The supernatant which contains the white cells and platelets is removed and centrifuged for 3 minutes at 1500 r p m The sediment is carefully resuspended by individual drops for washing in buffered physiological saline or Hanks solution to a volume of 30 ml A very homogeneous suspension should result from this procedure Washing and centrifugation is repeated and aliquots of the sediment are divided among 4 to 6 hemolysis tubes depending upon the number of leukocytes obtained These centrifuged suspensions provide washed leukocytic sediments ready for use They are kept in the refrigerator at $+4°$ C and can be used in succession one by one according to the needs of the experiment Thus one has at one s disposal for approximately 8 hours leukocytes in a perfect state of viability For the study of nuclear lysis we have also used leukocytes altered by being left 24 hours at room temperature

Slide Preparations

In order to make preparations for observations one takes up without aspiration by simple capillary action a mixture of leukocytes with LF serum (undiluted or diluted 1 2) in a Pasteur pipette whose end has been diamond cut exactly at right angles A very small drop is then placed on a perfectly clean slide and is covered with a 2 by mm cover slip It is important that this manipulation be well carried out in order to obtain good preparations If the drop is too big the cells remain rounded for a

long time and are difficult to observe If the drop is too small the leukocytes are compressed and die rapidly The space between cover slip and slide must be just sufficient to permit a leukocyte partially extended to move on its support without interference to its motion As a final step the preparations are sealed with paraffin The examinations are made at laboratory temperature In certain instances for the study of nuclear lysis test serum is added during the period of observation A very small drop of white cell suspension in physiological saline is placed between slide and cover slip and the preparation is sealed only on three sides Undiluted serum is added to the preparation at the desired moment on the fourth side In our more recent experiments we have utilized a gamma globulin extracted by Seligmann [51] from a precipitate prepared by the action of lupus serum on desoxyribonucleic acid of calf thymus This gamma globulin identified by ring tests with the aid of rabbit anti human gamma globulin serum is added by the same technique that we have just described either to leukocytes washed twice and suspended in physiologic saline or to leukocytes washed but resuspended in their own serum (fresh or heated at 56° C for 30 minutes)

Observations with Phase Contrast and Technique of Microcinematography

Our observations were made with a Wild M 10 microscope with Zeiss Winkel phase contrast equipment We have always used an oil immersion objective ×90 and a photographic ocular Zeiss ×4 or ×6 The micro cinematographic equipment was that which we have described earlier [43] The film used in these experiments was Duplo Pan Rapid of Gevaert having 17 Scheiner sensitivity to artificial light Photographs were taken as often as one per second and as seldom as one every 5 seconds The illumination was interrupted between pictures to avoid harmful effect of the illumination on the cells [6] The projection of the film at 16 frames a second gives an acceleration of the observed phenomena of 16 to 80 times It permits one to distinguish very easily the normal cytoplasmic movements in their accelerated form from the perfect immobility of dead portions of cytoplasm We will see the importance of this fact in the course of considering the results The photographs which illustrate the present paper are direct enlargements taken from the 16 mm negative film

Our findings have been essentially constant with only slight variations from one preparation to another whether the serum used was fresh or frozen for as long as 10 months whether it was obtained from a patient during an exacerbation or during remission whether the serum donors were or were not under treatment with cortisone whether the test leukocytes were furnished by donors of compatible or incompatible blood group and whether the test leukocytes came from normal individuals or

from patients with inflammatory disease The action of heparin in the concentration used and the utilization of polyvinylpyrrolidone as an accelerator of sedimentation have had no effect on the appearance of the specific phenomena Let us recall that the white cells are carefully washed The light used for photography has not affected the phenomena observed adversely as shown by control preparations left in darkness Finally the use of normal human serum in control preparations has never given rise to phenomena which could be interpreted as remotely resembling what we have observed with lupus serum

Results

Study of Nuclear Lysis

This is a rapid phenomenon which takes several seconds to several minutes to occur The most clear cut results were obtained by adding lupus serum under observation to altered leukocytes obtained either from normal or leukemic individuals At the moment of the addition of serum one observes in every case a contraction of short duration of both the cytoplasm and the nucleus the pattern of nuclear chromatin is then progressively effaced and the chromatin becomes homogeneous The lysis shown in Figures 1–4 was completed in approximately one minute The nuclear homogenization can occur in two phases With polymorphonuclears (Figures 5–9) one observes a violent contraction of the cells at the moment when one adds the serum (Figure 6) and the nucleus becomes homogenous (Figure 7) Then the cytoplasm swells again and the chromatin reappears (Figure 8) Finally the nucleus becomes homogenized again and increases in size (Figure 9) With lymphocytes (Figures 10–15) the same phenomenon is observed Figures 1 and 13 show that the homogenization of the nucleus may be reversible for a brief period Finally it is produced again and the lysis becomes definitive (Figures 14 and 15) In order to set aside the hypothesis that our observation could be an artifact due to a momentary loss of focus we have observed fixed points which remained clearly in focus throughout this change It is important to note that the contraction is not specific — it is produced also when one adds normal serum

When test serum is added to living leukocytes one can observe progressive lysis of the nuclei in cells which show signs of damage but are still alive (production of surface bubbling followed by abnormal solvation of the cytoplasm) An example is shown in Figures 16–19 which give a good idea of the nuclear lysis + points to the damaged nucleus We have also noted in the course of rosette formation that leukocytes in a perfect state of viability participate actively in the formation of the rosette and they can be suddenly affected by the lysing factor One observes

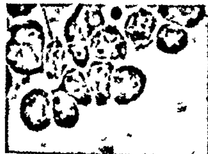


Figure 1 Before the test



Figure 2 Addition of I I serum

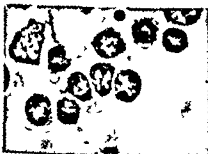


Figure 3 Twenty three second after addition



Figure 4 Tw[illegible] five s[illegible] after a[illegible]

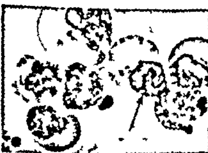


Figure 5 Before the test



Figure 6 Addition of I I [illegible]

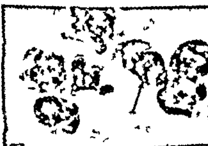


Figure 7 Four second after addition



Figure 8 Thirty [illegible] addition

then a progressive homogenization of the nucleus with loss of the normal chromatin structure This phenomenon is well illustrated in Figures 35-38 (N points to the damaged nucleus) It seems to us possible to affirm however that there exists a relation between the rapidity of nuclear lysis and the viability of the cell the more the cell is altered the more rapidly lysis occurs

It is easy to find in these preparations after 15 to 30 minutes numerous lysed nuclei still completely (Figure 20) or partially (Figures 21 and) surrounded by cytoplasm

Several facts of importance must be emphasized While some of the lysed nuclei of polymorphonuclear leukocytes may keep their lobulation most frequently the lobules swell and fuse Lysed nuclei unaccompanied by cytoplasm may be encountered, these are most often of lymphocytic origin The increase in size of the lysed nuclei is a fundamental phenomenon (Figures 3 and 4) This lysis must be of a peculiar type since it is followed by phagocytosis (Figure 25) (phagocytosis is never seen with lysed nuclei in control preparations) Further examples of these two aspects of the phenomenon are seen in Figures 26 27 and 28 The increase in nuclear volume is usually of the order of 3 to 4 times but may be greater Figure 29 shows an example of positive chemotaxis towards a lysed nucleus whose size is indeed quite unusual

In addition to the nuclear phenomena which dominate the picture one observes certain cytoplasmic changes Time lapse microcinematography facilitates the study of these phenomena which are difficult to observe directly The cell in contact with lupus serum, shows signs of damage The periphery of the cell shows violent bubbling, this later stops and the cytoplasmic granules show Brownian movement an evidence of solation of the hyaloplasm Finally a rapid jellying of the cytoplasm occurs and the granules become motionless and accumulate in the perinuclear region At the same time a liquid phase limited by a thin membrane is formed at the periphery of the cell In Figure 26 one can distinguish in the con cavity of the nucleus a dense granular mass made up of adherent, immobilized granules Similar but smaller masses can be seen all about the nucleus The whole is surrounded by a transparent zone limited by a delicate membrane Still later, this fine membrane disappears (Figure 8) and the liquid content is dispersed the granular formation persisting (Figures 20 1 and 22) It is to be noted that the increased volume of the nucleus frequently brings about rupture of the cytoplasmic band which surrounds it The nucleus then herniates to the exterior and finds itself in direct contact with the medium (Figure) We will see later the importance of this phenomenon

It is difficult to determine whether the nuclear or the cytoplasmic changes occur first It appears most frequently that the lesions of nucleus

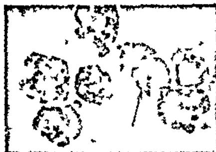


FIGURE 9 Eight minutes nine seconds after addition

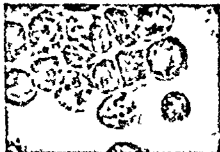


FIGURE 10 Before the test

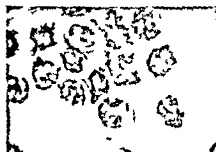


FIGURE 11 Addition of L E serum

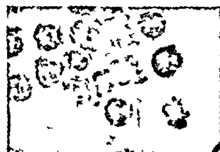


FIGURE 12 Seven seconds after addition

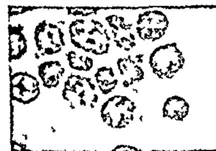
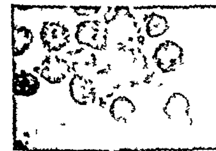


FIGURE 13 Fourteen seconds after addition



FIGURE 14 Twenty six seconds after addition





and cytoplasm appear simultaneously and evolve in parallel In any event important changes may still occur in the nucleus at a time when no further alteration of the cytoplasm is occurring

Study of Rosette Formation

Rosettes represent an active phenomenon They are the result of active chemotaxis of polymorphonuclears towards lysed nuclei (Figure 9) They do not represent a passive agglutination of living cells and lysed nuclei The films show that the movement of the polymorphonuclear cells is oriented This phenomenon is morphologically comparable to that seen when a suspension of the same cells in fresh serum is placed on a slide to which grains of potato starch have been previously fixed this phenomenon has been studied by Delaunay and Pages[7] Robineaux[41] Nelson and Lebrun[37] The latter demonstrated that starch is not devoid of immunological properties and that both complement and a specific antibody are necessary for its phagocytosis Since there is an evident similarity between rosette formation and adhesion of the polymorpho nuclears on the starch particles of big size (prepared from potatoes) it may be reasonably concluded that the close analogy which is noticed in the chemotactism with starch granules and L E bodies is probably suggestive of a mechanism of the same kind

The formation of rosettes is relatively slow requiring from 10 minutes to an hour It depends on the viability of the leukocytes and on their number in the part of the preparation where the lysed nuclei are located Lysis is a necessary but not sufficient condition for the formation of rosettes It is essential that the lysed nuclei be in actual contact with the medium -- in other words that the cytoplasms have been broken A nucleus completely surrounded by cytoplasm never gives rise to the formation of a rosette

In describing the nuclear lysis we mentioned an increase in nuclear volume This appears to occur inside the nuclear membrane (Figures 16–19 6– 8) When living motile polymorphonuclears encounter a lysed nucleus there is frequently produced a rupture of the nucleus followed immediately by extrusion of nuclear material In each case observed this apparently mechanical rupture was followed by the very rapid formation of a rosette several polymorphonuclear cells arriving in rapid succession to surround the ruptured nucleus The schematic drawing on page 380 depicts these events which are also illustrated in Figures 30–38

Figure 31 shows the compression of a lysed nucleus between two polymorphonuclears Figure 3 the rupture of the nucleus Figures 33–38 the progressive formation of the rosette Figure 38 shows that the cells in the rosette attempt to separate once the lysed mass has been subdivided and ingested Another example is shown in Figures 39–44 The homoge



Figure 17 The same as Figure 16 forty seconds later



Figure 18 The same as Figure 16 one minute forty seconds later



Figure 19 The same as Figure 16 three minutes forty seconds later

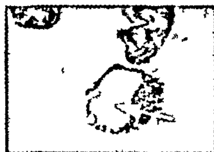


Figure 20

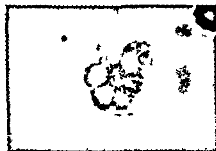


Figure 21



Figure 22

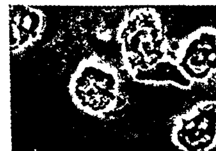
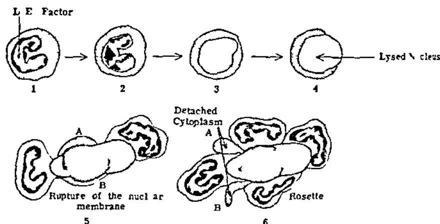


Figure 23



Figure 24



NUCLEAR LYSIS AND FORMATION OF ROSETTES

nization of the nucleus need not be complete at the time that the phenomenon begins In Figure 39 one can still distinguish the chromatin *structure at the moment that a phagocytic cell makes its first contact* with the nucleus As a last point the cytoplasmic fragments adherent to the lysed nucleus are frequently pushed aside and are not included in the rosette In the time lapse sequences one can readily recognize these dead fragments which are frequently completely detached from the lysed nucleus Their immobility is in contrast to the lively movements of nearby living cells Nevertheless they may be included in the rosette when they *are small and when the phagocytic leukocytes become attached to the nucleus and overflow them on both sides*

Study of the Formation of L E Cells

The L E. cell may be formed in different ways A living polymorphonuclear leukocyte may phagocytize a lysed lymphocyte nucleus Generally these nuclei have lost the thin rim of cytoplasm which normally surrounds them and are free in the medium The phagocytic mechanism is simple entirely comparable to that which is seen in the course of *bacterial phagocytosis of which we have made a dynamic study* earlier[9, 4] When one is dealing with lysed polymorphonuclear nuclei, the phenomenon is more complex Phagocytosis may involve an entire nucleus whose lobes are fused (Figures 45 and 46) In contrast phagocytosis may involve a single lobe when the lobes are not fused One can see this type of fragmentation clearly (Figures 47–49), it also gives rise to the formation of a typical L E. cell However it is the study of the phagocytosis of homogenized nuclei which are incompletely still surrounded by cytoplasm that has provided us with the most interesting

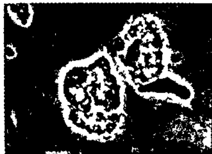


Figure 25



Figure 6

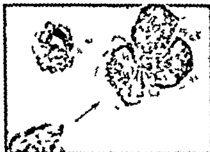


Figure 2

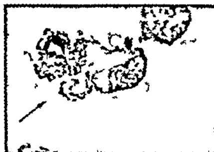


Figure 18



Figure 29

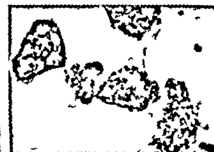


Figure 30



Figure 31 The same as Figure 30 two minutes thirty s conds later

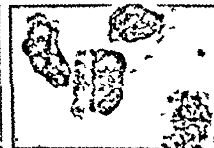
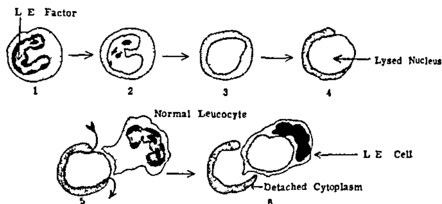


Figure 32 The same as Figure 30 five minutes, fifty seconds later



NUCLEAR LYSIS AND L E CELL FORMATION

observations In the above diagram the sequence of events is indicated schematically

Figures 50–53 are satisfactory representations of the process Two cells L and L_1 have lysed nuclei One sees the progressive extraction of nucleus I by the phagocyte P whose hyaloplasmic veils penetrate the interior of the damaged cell One sees clearly that the cytoplasm remains outside the phagocyte and is not absorbed (Figures 50 51 and 52) Figures 52 and 53 show another polymorphonuclear P_1 attacking the nucleus of the leukocyte I_1 at the precise point where this nucleus is bare of cytoplasm and in direct contact with the medium There results a veritable dissection of the nucleus which is detached from the cytoplasm by the hyaloplasmic veils of the phagocyte The indications are that this cleavage occurs in the nucleocytoplasmic interspace In this instance we lack certain evidence that the cytoplasm is completely rejected since it remains attached to the phagocyte The rejection of the cytoplasm is however established conclusively in the sequences represented in Figures 54–59 These figures show in succession the attack on nucleus I_2 by phagocyte P the separation of this nucleus from the cytoplasm C which surrounded it and its rejection and abandonment in the medium (Figure 59) It is to be noted that the ingested nucleus homogenous at the onset of phagocytosis has undergone a double transformation a cleavage into two fragments and a considerable secondary condensation These are phenomena that we have frequently observed

Comments

We will discuss our results from two points of view morphologic and immunologic

The morphologic aspects of the formation of the L E cell of Hargraves[11] have already been the subject of numerous studies These were



Figure 33 The same as Figure 30 twelve minutes thirty seconds later



Figure 34 The same as Fig fifteen minutes fifty second



Figure 35 The same as Figure 30 thirty minutes later

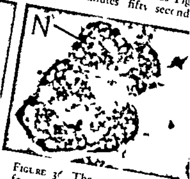


Figure 36 The same as Figure 3 forty minutes later



igure 37 The same as Figure 30 one our eighteen minutes later



Figure 38 The same as Figu hour fifty three minutes lat

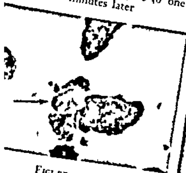


Figure 39



Fi

largely carried out with fixed and stained preparations Rebuck and Berman in 1950[38] reproduced the L E phenomenon in the skin of normal volunteers They described nonspecific cytoplasmic lesions in polymorphonuclear cells followed by swelling and homogenization of their nuclei The latter free in the medium were ingested by neutrophilic leukocytes They noted in some instances fragmentation of the lysed nuclei the 'L E bodies They described the occurrence of secondary changes in some of the host cells This appears to have been a significant observation

Numerous authors Dameshek and Bloom[5] Bessis and Tabuis[1] Moore, Weisberger and Bowerfind[35] advanced the hypothesis that the basic phenomenon was a fusion and autolysis of the nucleus and the cytoplasm together Finch Ross and Ebaugh[8] studying the action of heteroantiserums produced experimentally against granulocytes, obtained occasional pictures apparently identical with L E cells Nevertheless they noted that the polymorphonuclears were phagocytized intact and destroyed later whereas in the L E phenomenon the nuclear lysis is the preliminary event They also distinguished simple agglutination of cells brought about by the antileukocytes serum from the formation of rosettes regarding the latter however as a foreign body reaction of leukocytes to nuclear debris Similar experiments were carried out by Zimmerman Walsh and Heller[58] and similar conclusions were reached Miescher[29] with the use of heteroantiserums against thymus and spleen produced alterations in leukocytes resembling the L E phenomenon Nevertheless the cytoplasmic alterations were more marked and the nuclear changes more discrete Free nuclear bodies and rosette formation were rare This author in collaboration with Fauconnet and Beraud[32] suggested the hypothesis that the L E factor may be an antinuclear antibody He prepared guinea pig antibody against human leukocyte nuclei and was able to produce with this reagent acting on human leukocytes *in vitro*, massive nuclear alterations particularly in polymorphonuclears He describes pictures superimposable on the picture of the L E cell and concludes that the nucleus may act antigenically He reports similar findings with the use of antinucleoprotein antiserum[33]

It has seemed necessary to a number of authors to study the L E phenomenon in preparations of living leukocytes Thus Moyer and Fisher[36] studied supravitally stained preparations and Rohn and Bond in 1951[4, 45] made a microcinematographic study of supravitally stained preparations The first changes produced by the L E serum appear in the nuclei of heterophile polymorphonuclears swelling disintegration of the nucleus and its conversion into an amorphous mass surrounded by granulations Phagocytosis can only occur after a partial dissolution of the cytoplasm Bessis and Tabuis[1] studied living leukocytes *in vitro*

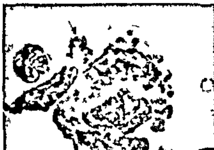


FIGURE 41 Same as Figure 39 eighteen minutes forty five seconds later



FIGURE 4 Same as Figure 39 twenty one minutes fifteen seconds later



FIGURE 43 Same as Figure 39 thirty three minutes forty five seconds later



FIGURE 44 Same as Figure 39 thirty five minutes forty-eight seconds later



FIGURE 45



FIGURE 46



FIGURE 47



FIGURE 48 Same as Figure 47 thirteen minutes twenty seconds later

with phase contrast microcinematography in the presence of pathologic or experimental antileukocyte serums. They describe appearances exactly comparable to the L.E. cell when phagocytosis involves leukocytes first lysed by the action of the antiserum. These authors nevertheless indicate that additional studies are needed before it can be asserted that the lesions produced by antileukocytic serums and those produced by lupus serum are identical.

The comparison of the results obtained with antileukocytic serum and our findings demonstrates important differences. Specifically, the formation of L.E. cells has nothing in common with the phagocytosis of whole cells. In contrast, meaningful results were obtained by Rebuck and Berman [3] and by Rohn and Bond [4, 4] by studying the L.E. phenomenon itself. It appears to us, nevertheless, that the study of living cells with phase contrast microcinematography represents the best technique for obtaining the maximum amount of information about this phenomenon. Very recently, Rifkind and Godman [23] have utilized phase contrast microscopy and interferometric microscopy. It is their results which may be most directly compared with ours. They studied in serial photographs the action of lupus serum on leukocytes by the technique of Davis and Eisenstein and of Lee [5] which employs damaged leukocytes as experimental substrate. They described a sudden homogenization of the nuclei a few seconds after the addition of test serum. These nuclei become of uniform density without losing the individuality of the lobes, of which each subsequently becomes an L.E. body. Some of these lobes may be expelled from the affected cells. They demonstrated a considerable increase in volume of these nuclei, to about three times the initial volume. They state that the cytoplasm is mechanically displaced by this increase in nuclear size and dispersed into the medium. Phagocytosis occurs if one incubates these preparations treated with L.E. serum with living leukocytes; the lysed nuclei, naked or still attached to cytoplasmic rests, are phagocytized. These authors emphasize the unimportance of the role of the cytoplasm in the evolution of the L.E. phenomenon; they regard it as being set aside from the moment of formation of homogenized nuclei. Certain of these points require discussion.

We agree with Rifkind and Godman as to the increase in volume of lysed nuclei, which appears to be a fundamental phenomenon. By interferometric microscopy they were able to demonstrate an actual increase in dry weight of these nuclei, which appeared to be explained by their earlier cytochemical observations: an influx of foreign protein accompanies or follows the "rupture" of the normal nuclear structure [11]. We have shown that the volume increase of nucleus, which is always quite sudden, is rapidly followed by events suggesting the appearance of a chemotactic stimulus (the rosette). We would suggest that it may repre



FIGURE 49 The same as Figure 47 twenty minutes later

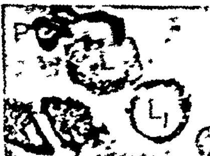


FIGURE 50



FIGURE 51



FIGURE 52

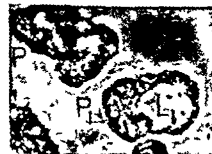


FIGURE 53



FIGURE 54



FIGURE 55 The same as Figure 54 five minutes fifty seconds later



FIGURE 56 Same as Figure 54 thirty four minutes a

sent the direct combination of a nuclear antigen with the L E factor, which many believe to be the corresponding antibody At present we believe that this antigen antibody complex possesses chemotactic power In our opinion this chemotaxis does not represent nor does the nuclear phagocytosis a banal reaction to foreign bodies we would give it an immunologic significance We would lay considerable stress on the im portance of the rejection of the cytoplasm and the specific character of the phagocytosis We differ with them as well on certain minor details, for example we find that lysed nuclear lobes can fuse and indeed regu larly do so More important, we have demonstrated that active leuko-cytes can be affected by the L E factor (see study of nuclear lysis—Figures 35–38) We would prefer to substitute for the notion of non viable cells[57] the concept of a gradient of viability We believe that the mechanism of action of this factor is more complex than the simple homogenization of the nucleus of a dead cell[54] Many of our observations were made possible by the use not of dead or altered cells but of suspensions of living leukocytes

We must now consider some histochemical properties of the L.E. nuclei It is already known that they retain the possibility to stain with Schiff's reagent after a moderate hydrolysis with chlorhydric acid that is Feulgen s reaction is positive On the other hand they have a lower affinity for methyl green than normal nuclei It is generally admitted that methyl green combines with the DNA molecules in stoichiometric proportionality [19, 2] and it has been also demonstrated "*in vitro* that the intensity of the coloration obtained with methyl green is in direct proportion with the degree of polymerization of DNA [19, 58] Therefore several authors have drawn the conclusion that since the intensity of the coloration with methyl green was decreased after the nuclei were homo genized by the L E factor the nuclear DNA was depolymerized [15, 1, 6]

Kurnick and his colleagues discussed the possibility of an enzymatic mechanism of the L E phenomenon [3, 4] They supposed that the normal inhibitor of DNase is destroyed so that the activation of the intracel lular DNase takes place[15] For the destruction of the inhibitor a special protease present in the L E serum is responsible Its penetration in the cell is achieved with the help of the L E factor [0, 21] This interesting hypothesis is built on a very simple analogy (as far as the decreased affin ity of the L E bodies for methyl green is concerned) [18, 12, 26] and on a negative finding that is the impossibility to demonstrate any depoly merizing action of the plasmatic DNase on the nuclei[23]

But Godman and Deitch[10] have established quite recently that it is possible to restore the normal affinity for methyl green in the L E nuclei after acetylation This is a proof that in the L E bodies the DNA is not depolymerized Here are their conclusions These data have been inter



Figure 57 Same as Figure 54, forty nine minutes, twenty two seconds later



Figure 58 Same as Figure 54, fifty five minutes, twenty six seconds later



Figure 59 Same as Figure 54 sixty two minutes twenty four seconds later



Figure 60

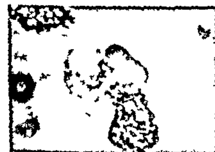


Figure 61 The same as Figure 60 one minute, thirty two seconds later

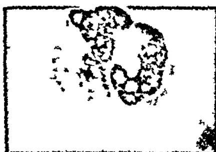


Figure 62 The same as Figure 60 four minutes, eight seconds later



Figure 63



Figure 64 The same as Figure 63 five minutes eight seconds later

preted to indicate (*a*) the presence in L E bodies of DNA associated proteins whose basic groups compete with the cationic dye for binding sites of DNA and so inhibit methyl green staining and (*b*) the DNA itself is not detectibly altered in state or degree of polymerization Photometric comparison of the mean Feulgen stainable DNA content per L E body with that of control nuclei showed that DNA is not lost in the L E transformation of nuclei

We may recall here that our studies in morphology substantiate the hypothesis that the fixation of proteins on the nucleus described by Godman and Deitch is an immunological phenomenon

Let us now consider the concept of a nuclear antigen in its relation to our morphologic observations The active agent has been found in the gamma globulin fraction of L E patients[14] Capelli[8] Marmont[27] Miescher[9 8 3] introduced the concept that an antinuclear antibody is responsible for the L E phenomenon Miescher later showed that the L E factor is absorbed by nuclei[3] and proposed the use of the antiglobulin consumption test as a useful technique for the identification of the factor[31] In 1954 Blix Iland and Stacey demonstrated that both nucleoproteins and DNA are antigenic but not the histones In 1957 Seligmann in a series of publications undertook the immunochemical study of lupus serums Having observed the presence in these serums of leukoprecipitins identified by the ring test Ouchterlony s technique and immunoelectrophoresis he was able to show that one of the leukocytic antigens concerned was DNA The precipitin reaction was observed equally with human animal or bacterial DNA[40 0] complement fixation was observed as well in a discussion of the nature of the serological substance responsible for this reaction he advanced arguments against its being a histone[1] Additional relevant data obtained at the same time or more recently should be mentioned

The studies of Mellors Ortega and Holman[25] Vazquez and Dixon Holborrow Weir and Johnson[16] in positive L E preparations the nuclei of leukocytes were the sites of localization of gamma globulin as demonstrated with the use of fluorescein labeled antibody

Experiments of Holman and Kunkel[17] suggesting that the L E factor has an affinity for nucleoprotein and that DNA is perhaps involved in the bond

Miescher s experiments[34] with the passive hemagglutination technique

And finally experiments on complement fixation with cell nuclei and DNA by Robbins and his collaborators[40] on the one hand and Ceppellini and his colleagues[4] on the other

More recently Seligmann[5] seems to have established that the substance in L E serum which precipitates with DNA is an antibody With the use of the same technique already described (see page 37) this sub

stance produces in the presence of normal serum a typical L E phenomenon (Seligmann and Robineaux[33]) These findings are illustrated in Figures 60–6 and 63–64 There were reproduced nuclear lysis with increase in nuclear volume and rupture of the cytoplasm positive chemotaxis of living leukocytes with the formation of rosettes phagocytosis of the entire nuclear mass or part of the nucleus with the formation of L E cells and the rejection of detached cytoplasmic fragments Control preparations (for details see our publication) never gave rise to any of these phenomena The isolated gamma globulin active in the presence of fresh serum proved inactive in saline or in the presence of heated normal human serum an observation which suggests the participation in the phenomenon of substance(s) with properties similar to those of complement

Conclusions

The morphologic observations presented in Figures 1 to 64 and the two diagrams appear to us solid arguments in favor of the hypothesis of an immunologic origin for the L E phenomenon They imply that DNA or perhaps desoxyribonucleoprotein can act as an antigen and give rise to the formation of specific antibodies This conclusion follows from the precipitation reaction seen with L E serum and purified DNA and depends obviously on the purity of the antigen used

Summary

1 A study of the effects of the serums of patients treated for acute disseminated lupus erythematosus on living normal human leukocytes was made with the technique of time lapse phase contrast microcinematography

Nuclear lysis chemotaxis and phagocytosis have been specially considered

3 The *nuclear lysis* begins very rapidly several seconds or minutes after the serum has been added The interval varies with the vitality of the cells The lysis is extensive in character and the nuclei were observed to increase 3 or 4 times their original size Further on the polylobular lysed nuclei fuse together The lysis of the nuclei which generally appears as a simple phenomenon may occur in several steps

4 On the contrary *chemotaxis* appears slowly (a few minutes to one hour) A careful study of the chronology shows that the formation of rosettes is facilitated by the rupture of the nucleus This is a proof that the process of rosette formation involves the participation of the internal constituents of the nucleus The nuclei of the living cells forming the rosette can also be affected by the lytic factor

5 *Phagocytosis* is also a relatively slow process (a few minutes to one hour) It may occur with a fraction or even a particle of a lysed nucleus The pictures demonstrate very clearly that the cytoplasm surrounding the lysed nucleus is separated detached and pushed aside The phagocytosis is exclusively nuclear Phagocytization of the cytoplasm has never been observed It may be concluded from these observations that the opsonin responsible for phagocytosis is strictly antinuclear

6 A comparison of the results obtained with an experimental anti leukocytic serum against normal human leukocytes shows important differences with the L E phenomenon

7 The correlation between the morphological findings and the immunological conception of a nuclear antigen in the mechanism of lupus erythematosus must be seriously considered

8 The effect on normal cells of a gamma globulin extracted from the precipitate formed by L E serum treated with DNA has been investigated The gamma globulin proved to act as an antibody against DNA it determined lysis and opsonization of the nuclei It was able to induce a typical L E phenomenon with human normal fresh serum and human living leukocytes

ACKNOWLEDGMENTS

The author is greatly indebted to Professor Raoul Kourilsky for his unfailing aid and encouragement He is most grateful to Dr Byron Waksman for kindly translating this paper Acknowledgments are given to Pr A Lemaire Pr Ag Laplane Pr Ag Delbarre Dr J Dausset Dr J Debray Dr B Dreyfus Dr F Basset Dr M Seligmann for generously providing L E serums

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DESIGNATED DISCUSSION

STUART C FINCH (New Haven Connecticut) Discussion of these beautifully presented papers and Dr Robineaux's beautiful film probably constitutes somewhat of an anticlimax but the material presented has raised some important questions Any consideration of autoantibody production or of its clinical significance moves one into a field of controversy almost as great as that which shrouds the production and identification of antiantibody

The autoimmune concept of disease, however, has received considerable support from the papers presented here this morning Evidence for this concept is growing but still remains quite unproven in man The general etiologic mechanisms of autoantibody production seem of sufficient importance to justify their brief review — even at the risk of being somewhat repetitious As has been mentioned the concept of autoantibody production is contrary to basic immunologic principles — that is the host's antibody producing mechanism is incapable of immunologic response to its own *normal* tissue Thus the detection of antibody in the host with specificity for its own tissue means that alterations in antigen structures antigen antibody response or in antibody production have occurred Plasma globulin in the form of autoantibody or reacting as autoantibody may evolve in one of several ways

Physical or chemical alteration of normal tissue (antigen) so that the antibody produced reacts with either the altered or normal native tissue protein The importance of chemical conjugation to protein and physical modification of protein to enhance antigenicity was emphasized by Landsteiner Landsteiner and Chase and others a number of years ago It is not difficult to visualize the development of such tissue protein modifications in man following the injection or inhalation of many exogenous compounds with obscure metabolic degradation pathways Furthermore it is not unlikely that individual hereditary or acquired peculiarities in tissue metabolism may predispose to these linkages in a fashion similar to those observed with primaquin and other drugs in a small segment of the colored population In the absence of drug ingestion it is not unlikely that disseminated neoplasia with or without virus is capable of rendering normal host tissue antigenic Again it should be emphasized however that none of these mechanisms has been clearly responsible for autoimmune disease in man

In our own laboratory we have been unsuccessful in producing autoantibody in guinea pigs and rabbits following autologous injections of erythrocytes and leukocytes modified by weak acid heat denaturation diazo conjugation and incorporation with Freund's adjuvant Intraperi

toneal subcutaneous and intradermal injection sites all were without success As was mentioned by Dr Swisher similar studies by others have shown a weakly positive antiglobulin reaction but no evidence of erythrocyte hemolysis We also have studied a number of patients subjected to lumbar splanchnicectomy operations following which there regularly occurs retroperitoneal extravasation of from 100 to 200 ml of blood During the next several months none of these patients developed positive Coombs tests or alterations in either osmotic or mechanical fragility of their erythrocytes

The *development of auto antibody to injections of normal ocular* reproductive neurologic and thyroid tissues in experimental animals seems contrary to the concept that tissue change is necessary but alteration of their structures through mechanical manipulation remains an excellent possible explanation for their antigenicity

Failure with some of the aforementioned experiments may have resulted because the serum reactive protein in these diseases is not true autoantibody but is strongly *cross reacting antibody formed against closely related abnormal protein* In this situation autoinjections of normal or altered target tissue would fail to evoke an immunologic response It would be virtually impossible to test this system experimentally since the antigen would be the abnormal tissue of or would be produced by some associated obscure disease Dr Swisher s report demonstrates the frequency with which autoimmune hemolytic disease develops in patients with lymphomas and leukemias which opens one possible avenue for further study Cross reacting antibody of this type would remain classified as autoantibody until the appropriate abnormal or new antigen is identified

The third intriguing possibility is that *natural or acquired isoantibody begins reacting with normal host tissue* This might result from (*a*) alteration of preformed antibody or (*b*) the removal or destruction of normal serum inhibitors through disease It is very probable that even in normal circumstances the red cell is accepting some serum (antibody) globulin The presence of such globulin coating of normal erythrocytes has been demonstrated by Stratton and Jones with antiglobulin absorption studies and in our laboratory studies done in collaboration with Drs Danaher and Friou have demonstrated this with the fluorescein antiglobulin tests The exciting observations by Dr Robert Nelson implicating the surface of the erythrocyte in certain antigen antibody reactions through immune adherence also indicate that antibody globulins normally constitute an important part of the red cell membrane Observations with leukocytes suggest that the red cell is not unique in this respect The importance of antibody globulin in delayed hypersensitivity passive transfer reactions is well established Additional fluorescein antiglobulin studies in our labo

ratory indicate the abundance of normal globulins in or on the cytoplasm of these cells There also are those who have shown either impairment of leukocytic function or their actual destruction following incubation with certain protein antigens

The metabolic cycle of these antibody globulins on or in these cells is obscure but it is clear that piling up of surface proteins not infrequently affects cell survival adversely This could occur either as the result of changes in serum (antibody) globulin structure or through the destruction of normal inhibitors due to underlying disease of another type

Other important mechanisms to be considered are those involving *derangement of the antibody producing mechanism* Altered responsiveness might result in any of the following

(*a*) The production of abnormal proteins which have an affinity for and destroy normal host tissue and simulate autoantibody in every respect

(*b*) The production of antibody against tissue previously nonantigenic

(*c*) A tremendous quantitative antibody globulin overproduction due to increased reactivity of the reticuloendothelial tissue This would promote increased cellular deposition of protein acting as antibody

It is well known that in many neoplastic diseases myriads of peculiar serum proteins are produced—probably not on an immunologic basis but probably due to diseases involving the reticuloendothelial system (lupus globulin macroglobulin Bence-Jones protein etc) The physical characteristics of these proteins are so abnormal that their reactivity as an autoantibody is not difficult to visualize The production of only slightly altered normal antibody might permit immunologic reactions with normal tissue to take place Against the concept that hyperreactivity of the antibody producing mechanism is responsible are the observations that autoimmune hemolytic anemia commonly occurs in lymphomatous and leukemic disorders In these diseases antibody response to a specific antigen challenge is poor Another bit of conflicting evidence is the observation that acquired hemolytic anemia with positive Coombs test may develop in the presence of hypogammaglobulinemia but rarely if ever develops in multiple myeloma

The final important general mechanism to be considered is one which embraces the concept that certain *normal host tissues challenge antibody producing tissue only when certain barriers are removed* This concept would be in accord with the observations that intra- or subcutaneous injections of ocular neurologic reproductive and thyroid tissues evoke autoantibody response with destruction of in situ structures The nature of these tissues makes this very feasible but it is difficult to justify this concept with the production of erythrocyte autoantibody Erythrocytes constantly are in intimate contact with plasma cells lymphocytes mono-

cytes and tissue reticuloendothelial cells during their life cycle and the introduction of immunologically incompatible erythrocytes rapidly evokes antibody response These inconsistencies must be justified before this attractive concept can be accepted Perhaps the suggestion of Dr Swisher's that a combination of altered antigen structure and antibody response is necessary for autoantibody production is the most reasonable

There exist a number of disparities between clinical and experimental autoantibody production Of the four normal body structures (thyroid lens nerve and sperm) which experimentally have produced autoantibody and pathologic target organ change there is clinical correlation only with disease of the thyroid At the present time one can only speculate as to the importance of antibody in the development of disorders of the lens (lenticular opacification and atrophy) reproductive organs (testicular atrophy and aspermatogenesis) and neurologic tissues (aseptic meningitis encephalomyelitis neuronitis and neuritis etc) This disparity probably is more apparent than real since only limited clinical immunologic studies in these disorders have been made Methodology also may have played an important role In most clinical states attention is directed to finding free or circulating antibody Limited attention has been paid to the detection of antibody on the target tissue such as occurs in autoimmune hemolytic anemia The application of similar techniques (i e fluorescein antiglobulin test Coombs consumption I[131] antiglobulin etc) to other tissues in a similar fashion might yield rewarding results The other important disparity which exists is the failure to experimentally reproduce certain human autoimmune disorders (acquired hemolytic anemia and idiopathic thrombocytopenic purpura)

The unusual observations described by Dr Roitt in chronic thyroiditis and other thyroid disorders certainly constitute strong evidence for the autoantibody hypothesis of disease As not infrequently happens however that which initially appears quite clear cut becomes considerably more complex when studied in detail Dr Ernest Witebsky and his associates have reported on observations in which saline extracts of thyroid or purified thyroglobulin plus Freund's adjuvant were injected intradermally into dogs rabbits and guinea pigs Using techniques similar to those described today about 40 per cent of the rabbits and guinea pigs were shown to have circulating antibodies In none of the dogs was there circulating antibody but in many of the dogs and other animals studied the histologic lesion of chronic thyroiditis was found In man they have demonstrated circulating thyroid antibody in many with chronic thyroiditis and circulating thyroglobulin in a few Other pertinent experimental studies are those of Kabat *et al* in which an allergic encephalomyelitis was produced in rabbits following injections of rabbit brain

emulsified with Freund s adjuvant Dr Jules Freund has described the development of aspermatogenesis in guinea pigs following immunization with spermatozoa of the same species Lenticular opacification and atrophy also have developed in animals injected with lens tissue from the same species These observations emphasize the importance of persistent search for similar mechanisms responsible for disease in man Of particular importance would be a number of etiologically obscure neurologic disorders (e g encephalomyelitis aseptic meningitis acute infectious neuronitis etc) The probability that autoantibody localized to the target tissue rather than in detectable serum quantities must be appreciated I should like to ask Dr Roitt a few questions Is it not possible that mechanical trauma to the thyroid might release thyroglobulin into the circulation and initiate the autoimmune process? Have studies of this type been tried? Why does intact thyroglobulin reach antibody forming tissues by means of the blood plasma without enzymatic disruption? Have any attempts been made to stimulate antibody production with any of the subcellular fractions?

Dr Swisher s broad experience with acquired hemolytic anemia was reflected in his fine presentation of the clinical experimental and etiologic aspects of this disorder Many of the questions which he raised are those to which we would most like the answers I will not ask these questions again since I am sure that he would have given us the answers if the information were available

Dr Holman s imaginative approach to the characterization of the lupus factor is most commendable Again these studies are illustrative of the complexities which have evolved from critical investigation of an abnormal serum factor Even if his evidence for incriminating nuclear autoantibody as the nuclear lytic factor is substantiated it appears that the abnormal gamma globulins produced in disseminated lupus show much individual variation Perhaps the disorder should be included in that group of disorders characterized by dysproteinemia Our initial interest in this field was stimulated when we demonstrated that when heterologous leukocyte antiserum was incubated with normal human blood cells resembling lupus cells evolved Soon it was recognized however that whole leukocytes were phagocytized and then lysed when exposed to this serum in contrast to the nuclear lysis with subsequent phagocytosis which occurs with lupus factor Antibody to whole leukocytes is stimuated primarily by cytoplasmic rather than nuclear components The experiments by Dr Peter Miescher in which he demonstrated the specificity of the lupus factor to isolated cell nuclei by absorption and Coombs consumption techniques prompted our application of the fluorescein antiglobulin test for demonstration of the localization of lupus globulin on cell nuclei These studies performed in collaboration with Drs George Friou and

Katherine Detre were initiated almost simultaneously with those of a similar nature in Dr Holman s laboratory Following incubation of human leukocytes mouse splenic cells and isolated calf thymus nuclei with human lupus serum bright nuclear fluorescence developed when these washed cells were exposed to fluorescein conjugated anti human globulin The reaction was positive in 7 of 28 lupus serum samples tested More recently 13 positive tests developed upon testing the sera of 23 lupus patients in which only 6 had positive lupus induction tests A few weakly positive reactions have been observed in some related disorders but the intensity of these reactions has not been comparable to those observed with lupus serum These studies provide strong additional evidence for the specific affinity of lupus globulin for nuclear protein

Dr Holman s studies on complement give additional support to the autoimmunologic concept of disseminated lupus Testing with separate nuclear components has yielded varied results which should be more meaningful as these studies progress In the presence of complement inhibiting concentrations of EDTA we have noted cessation of L E cell and rosettes formation but no inhibition of nuclear lysis These studies suggest that without complement phagocytosis but not nucleolysis is inhibited It would be of considerable interest to find out whether or not Dr Holman has had an opportunity to incriminate any particular portion of complement in these studies I should also like to ask whether or not Dr Holman would care to speculate on the possible etiologic mechanism in disseminated lupus erythematosus

Dr Robineaux s film is instructive and beautiful beyond description The mechanism of L E cell formation is clearly shown but I should like to ask Dr Robineaux just why the nuclei of some leukocytes lyse so quickly while others seem uninfluenced by the lupus factor and actively phagocytize Finally I should like to congratulate all of the speakers on the clarity of their presentations and for the conservatism expressed in the interpretation of their data

GENERAL DISCUSSION

(Dr Merrill Chase assumed the Chair)

CHAIRMAN CHASE Dr Robineaux before leaving France specified that his pictures should be projected at 16 frames a second but in our very modern auditorium we were unable to provide a speed less than 24 frames a second since now we are accustomed only to sound films Nonetheless I believe that you will have acquired something of particular value one must pay tribute to a wonderful piece of cinematography We are grateful to you Dr Robineaux

Before we allow the preceding speakers to answer the various points that have been raised Dr Robert White will show several slides bearing on the question of thyroid antibodies

ROBERT G WHITE (London England) I should like to reinforce the remarks of Dr Roitt by giving you a picture of the distribution of the antigen in the thyroid gland related to the antibody which occurs in the serum of patients with Hashimoto s disease We can study this antigen distribution by treating frozen sections of thyroid with a fluorescein conjugate of the globulin fraction of the patient s serum

Figure 1 shows the result with a thyroidectomy specimen from a case of thyrotoxicosis This shows the presence of antigen within acini and also within the acinar cells often as globules between the nucleus and the acinar space The antigen which is thus demonstrated appears to be that referred to by Dr Roitt as the antigen of the precipitin or hemagglutinin system This can be shown using sera which give a positive precipitin test and a negative complement fixation test The antigen of the comple

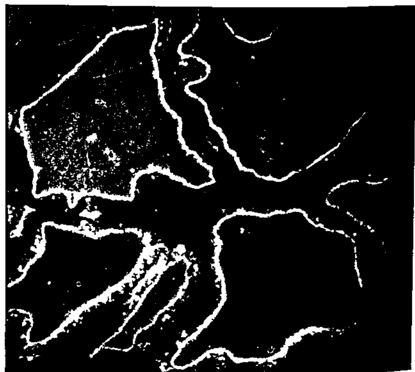


FIGURE 1 Fluorescence micrograph Thyroidectomy thyroid frozen section treated with fluorescein conjugate of globulin fraction of serum of patient with Hashimoto s disease Bright areas indicate distribution of antigen within acini and acinar lining cells

ment fixing system might similarly be localized by the use of a serum showing a high titer of complement fixing antibody with negative precipitin and tanned erythrocyte tests This approach shows no localization to intraacinar colloid and in the case of this antigen we are not yet sure of its distribution which may be difficult to determine owing to low concentration

The difficulties posed by isoantigens and antibodies in this work may be avoided by using a fluorescein conjugate on the frozen section of a thyroid taken from the same patient as the serum This would be a patient with Hashimoto s disease Figure shows similar localization to the intra



FIGURE 2 Fluorescence micrograph Thyroidectomy specimen from case of Hashimoto s disease Frozen section treated with fluorescein conjugate of globulin fraction of the patient s own serum Bright areas indicate antigen within acini and its escape outside among and within the surrounding granuloma cells

acinar colloid The antigen in this case extends out beyond the acinar cells into close contact with the surrounding granuloma cells Some is present within these cells These appearances show how the release of antigen can provide a continuous stimulus for antibody production However these data do not allow an understanding of the mode of initiation of the antibody response

ZOLTAN OVARY (Baltimore Maryland) I should like to report some of the experiments which we made with Dr Witebsky using passive cutaneous anaphylaxis (PCA) to detect thyroid autoantibody in humans and also in animals I report only the cases with humans













FIGURE 1 Human Serum 1/200 1/100 1/50 Rabbit Serum 1/2000 1/1000 1/500

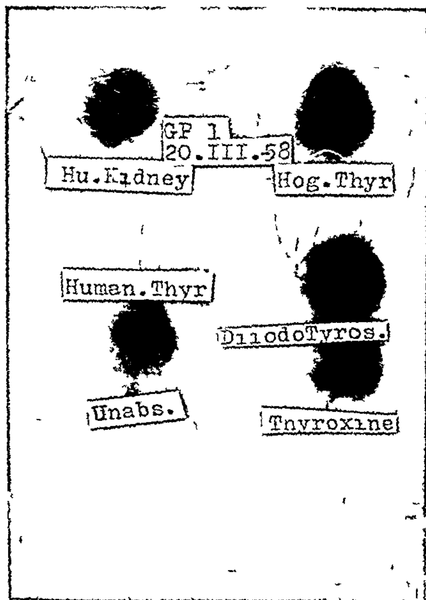


FIGURE 2 Absorbed with human kidney human thyroid unabsorbed absorbed with hog thyroid diiodotyrosine thyroxine

The technique is very simple We inject the antibody (the serum) in the skin of the guinea pig and 3 or 4 hours later we challenge the animal with a saline extract of thyroid and a dye

The first slide (Figure 1) shows the skin of a guinea pig which received on one side three dilutions of rabbit antibody against human thyroid and on the other side three dilutions of human thyroid autoantibody It is evident that the human serum gave also positive reactions Controls (injected only with dye and no antigen) gave negative results Human thyroid autoantibody can therefore be detected by PCA

To show the specificity of this reaction we made absorption experiments Table I shows such an experiment It is evident that only human

TABLE I PCA IN GUINEA PIG WITH HUMAN ANTITHYROID AUTOANTIBODY

Treatment	Result
Unabsorbed	++
Absorbed with 500 gamma hog thyroid	++
Absorbed with 500 gamma diiodotyrosine	++
Absorbed with 500 gamma thyroxine (1 d)	++
Absorbed with 0 1 ml human kidney extract (1 10)	++
Absorbed with 0 1 ml human thyroid extract (1 100)	−
Absorbed with 3 gamma N human thyroglobulin	−

thyroid extract or human thyroglobulin can absorb the antibody The other antigens (hog thyroid human kidney diiodotyrosine thyroxine) do not even diminish the reaction though relatively more of these antigens were used The second slide (Figure) illustrates such an experiment

DR HOLMAN We have not attempted to determine which components of complement are involved in these reactions

The problem of inducing immunization to nuclear constituents experimentally in animals is a very interesting one and we are starting such studies In the disease the appearance of antinuclear antibodies might be a consequence of an alteration in the patient s nuclear constituents making them antigenic or of an abnormal response by the patient s immune mechanism resulting in antibodies to normal nuclear constituents We have thus far failed to obtain complement fixing factors against nucleic acid molecules in rabbits immunized with normal nuclear constituents We

have however obtained some evidence for factors which react with the non nucleic acid portions of the nucleus

In the disease it is perhaps more likely that there is a derangement of the antibody producing mechanism in view of the fact that lupus patients appear to form other antibodies with great facility With regard to the induction of an abnormal immune response experimentally a clue is provided by the fact that the drug hydralazine can give rise to a syndrome similar to systemic lupus erythematosus in patients with the appearance of the lupus cells inducing factor in the serum We have been unable to produce a disease syndrome or any of the reactive serum constituents of lupus in rabbits by giving them large doses of hydralazine for long periods of time

One interesting but totally unsubstantial speculation is that in systemic lupus erythematosus something happens to the antibody-producing mechanism resulting in circulating serum constituents which are actually imperfect antibodies that is to say antibody molecules which have escaped prematurely from the synthesizing or imprinting mechanism and which still retain some affinity for the nucleic acid backbone of the cell nucleus or cytoplasm which is intimately involved in protein synthesis There is of course no evidence to support this idea

Dr. Roitt With respect to Owen and McConahey s demonstration of a thyroglobulin like protein in the serum of patients with Hashimoto s thyroiditis three possibilities come to mind One is that thyroglobulin may be present as an antigen antibody complex in the serum. Another possibility is that these patients may have only complement fixing anti bodies not directed against thyroglobulin itself so that follicular disrup tion occurring in the course of the disease would lead to leakage of thyroglobulin without any subsequent combination with antibody Alter-natively an iodinated protein other than thyroglobulin may be involved

We have not tried to initiate autoantibody formation by traumatizing the thyroid gland in animals but I should like to cite the evidence pro-vided by patients with subacute thyroiditis of viral etiology in which there is extensive damage to thyroid follicles thyroglobulin can be detected in the circulation and subsequently autoantibodies appear Administration of I^{131} in therapeutic doses results in a release of thyroid proteins from the gland and Lindsay s studies have shown that such treatment may lead to histological changes which resemble the lesions in Hashimoto s thyroiditis

We agree with Dr Finch that it would be of considerable interest to study the effect of autoimmunization with thyroid microsome fractions

Our results on the species specificity of the thyroglobulin precipitin are in accord with those of Dr Ovary We found cross reactions of the precipitating Hashimoto antibody only with extracts of the thyroid glands of the monkey and chimpanzee but not with those from a number of other mammalian species

DR ROBINEAUX There are two questions On the one hand not all nuclei are lysed on the other hand not all lysed nuclei are phagocytized

To answer the first question Snapper has demonstrated that nuclei which are already damaged are much more readily affected by the L.E. factor This is undoubtedly true but we have been able to show in our film that living cells can be attacked or damaged by the L.E. factor There probably is such a thing as a gradient of vitality, and one supposes that certain cells can be affected by the lupus factor from the moment that their vitality goes below some threshold level

In answer to the second question I believe the complex of antigen antibody is able to bring about positive chemotaxis and the phagocytosis of the antigen antibody complex

Not all lysed nuclei are phagocytized but we have shown in the film that once the nuclear membrane has been broken then chemotaxis in the formation of rosettes occurs extremely rapidly

We think as a possibility that the antibody has united with the totality of nuclear structures We suggest that its penetration is made possible by the porous nuclear membrane (as seen on electron microphotographies) Thereafter the nuclear membrane would break down and place the opsonized nuclear structures directly in contact with the medium, allowing phagocytosis

GUY A VOISIN (Paris France) I have been extremely interested by the fascinating papers presented this morning on the subject of autoantibodies However they were especially concerned with human diseases a field which seldom allows a study of the general problems that are involved These problems concerning the general characteristics of autoantigens and autoantibodies and the general concept of autosensitization can be best studied by an experimental approach and seem to me to be very important For example in the case of autoantigens why are spermatozoa myelin lens and some other organs or substances spontaneously auto antigenic? Is there a common denominator to the immunological specificity of the different autoantigens? What could be their nature?

A consideration of autoantibodies presents other problems What is their specificity and their nature as compared to isoantibodies and hetero antibodies? What can be the relationship between autoantibodies and experiment autosensitization diseases

I have been studying these various problems for several years but I will restrict my discussion today to the problem of immunological specificity of autoantigen autoantibody systems Schematically any antigen from a living organism falls under one of these categories *individual specific, strain specific, species specific organ specific* Group specific antigens and heterogenetic antigens are particular cases related to the preceding ones I think that autoantigens are organ specific, but we must explain what we mean by organ specific We mean an antigen that is not shared by all the organs or all the cells or all the tissues of an organism but which is characteristic of an organ a tissue or a type or system of cells In addition it is shared by the corresponding organs tissues or cells in any individual at least of the same species (and often of some other species as well) It seems likely that these antigens play a particular and precise role in the function of the organism It would even be better to speak of *function* specificity than of *organ* specificity

What is the evidence for organ or function specificity of autoantigens Two sets of experimental evidence support this view

The first one comes from known experiments on autosensitization for example allergic encephalomyelitis can be induced in a monkey by injecting it with rabbit brain monkey brain or part of its own brain Actually nothing but brain or spinal cord or extracts of them can induce this phenomenon and no other organs are involved in the experimental disease

The second comes from experiments that we have especially planned to study the question of organ specificity of autoantigens We will refer here only to the part of our experiments dealing with spermatozoa and with passive cutaneous anaphylaxis utilized as a test to demonstrate serum antibodies

For the purpose of immunization guinea pigs were injected with testis homogenates and/or spermatozoa mixed with Freund s adjuvants Testes came from the injected guinea pig from other guinea pigs or from other animals (rabbits rats and mice) After a suitable period of time (technical procedures are described in detail elsewhere) serum was taken from the guinea pigs and tested against testes or spermatozoa extracts of the serum donor guinea pig or other guinea pigs or other animals (rabbits) according to the passive cutaneous anaphylaxis test procedure The results can be seen in the table on page 410

We obtained spermatozoa autoantibodies* by injecting guinea pigs with either their own testis (or spermatozoa) testis (or spermatozoa) from other guinea pigs or testis (and spermatozoa) from rabbits rats

* Autoantibodies being — we must insist on the definition — antibodies elaborated in a given organism and able to react immunologically with a constituent of the same organism

TABLE I ORGAN SPECIFICITY OF AUTOANTIGEN AUTOANTIBODY SYSTEMS AS SHOWN BY THE EXAMINATION OF SERA BY PASSIVE CUTANEOUS ANAPHYLAXIS (PCA)*

PCA Elicited with Evans Blue Mixed with Extracts of	Sera Obtained from Guinea Pigs Immunized with the Following Tissues: Testis Spermatozoa and Source			Kidney and Source			None†
	Autologous	Homologous	Heterologous	Autologous	Homologous	Heterologous	
Spermatozoa							
Autologous	+	+		—	—		—
Homologous	+	+	+	—	—	—	—
Heterologous	+	+					—
Kidney							
Autologous	—						—
Homologous	—	—	—		±		—
Saline only	—	—	—	—	—		—

Homogenates of tissues of the same guinea pig (autologous) or of other guinea pigs (homologous) or of rabbits mice or rats (heterologous) were emulsified in Freund s adjuvant and injected Sera from the guinea pigs were injected into the skin of young normal guinea pigs 6 to 16 hours later the challenge antigenic extract (or saline) was mixed with Evans Blue and injected intravenously The test extracts are considered to be autologous homologous or heterologous in relation to the individual guinea pig that provided the serum used in the passive cutaneous anaphylaxis test

Legend + positive reactions in most recipient animals ± positive results not encountered frequently (3 out of 17) — consistently negative findings Not all combinations were explored

† Some sera were taken from guinea pigs that had received injections of Freund s adjuvant emulsified with saline only

and mice (In the last case for technical convenience the utilized test antigen was not *autologous* but *homologous*, as shown in Table I)

On the other hand sera from guinea pigs injected with their own testis (or spermatozoa) were able to react with spermatozoa extracts coming from their own testis or spermatozoa from other guinea pigs or spermatozoa from rabbits We are therefore authorized to say (at least in the case of spermatozoa) that autoantibodies can be elicited by injections of autoantigens isoantigens or even (sometimes) heteroantigens provided that the immunizing antigen comes from the same organ (the same functional mechanism) in each case and correlatively that autoantigens when injected elicit the formation of antibodies able to act as autologous isologous or even (sometimes) heterologous antibodies What do these results mean if not that the autoantigen autoantibody system is strictly organ or function specific that it has nothing to do with individual specificity and to a certain extent with species specificity?

This is true at least for spermatozoa but there
think that it holds true for any kind of autoantigen-
To take only a few examples experiments on lens a:
since lens *from* several species induces when injecte
tion of antibodies able to react with rabbit lens extr:
other organ extract In the case of blood cell disease:
these antibodies are able to react not only with th
but with corresponding cells of any normal person '
the cases of brain myelin and allergic encephal
extremely suggestive also However in the latter c
(autoantibodies) are not thought to play an activ
which would be caused by cell bound antibodies o:
However from the known facts what we said abou
be true for autosensitization in general without re
hypersensitivity involved since it is well known
specificity is a general rule for the delayed typ
(without detectable serum antibodies) as well as fo
of hypersensitivity (with serum antibodies)

All those facts together show that autoantigen :
and autosensitization phenomena are organ or funct
are not (and from a theoretical standpoint cannot l
and that they are not species specific Even if an
found in only one species, it still would be *organ* or
not *species specific* in the sense that it would be r
area or a certain function not belonging to the w
substance(s) responsible for species specificity
hereditary genes) do extend to the whole organism
belonging to the same species and cannot lie in only
function of an organism

This view on organ specificity (or function spe
tization is only part of a broader concept con
categories of antigen antibody systems among livin
antibodies or antigen antibody systems can be cl
their biological specificity as individual strain
specific

Antigens and antibodies can also be classified acco
relationship between the animal which provides the
the antibody) and the animal in which the antigen
an antibody (or in which the antibody reacts wit
According to this relationship antigens and antibo
the following categories *heterologous* where the
producing animal is of a species different from th
corresponding antibody is elaborated (or in wh

antigen reacts) *homologous*, where the two animals ('donor' animal and 'reactive' animal) are of the same species, but not of the same strain, *isologous*, where the two animals are of the same strain, and *autologous*, where 'donor' and 'reactive' are the same animal

Examination of the known facts in agreement with theoretical views leads to the following relationships between the two preceding types of classification

TABLE II Two Classifications of Tissue Antigens and Corresponding Antibodies in Higher Organisms and the Interrelationships Between Them

Heterologous	Species specific
Homologous	Strain specific
Isologous	Individual specific
Autologous	Organ specific

The connecting lines show that an antigen (or antibody) in the left hand column can possess the degrees of specificity indicated in the right hand column and that an antigen (or antibody) in the right hand column can have the origins indicated in the left hand column

Without translating all the elements of this table into full sentences I should like to stress three points from it first, autoantigen autoantibody systems are only organ specific (in the already defined sense), second species specific antigen antibody systems are only heterologous and third when strains are highly inbred and tend to become 'pure' isologous systems tend to become similar to autologous and strain specificity tends to become close to individual specificity (the extreme case being that of identical twins) as shown by intrastrain grafts (isografts)

These considerations have also other implications and other problems of general interest rise from the question of autosensitization but I cannot even summarize them in the short time and space allowed here

I wish to emphasize that autosensitization is not merely a collection of particular and amazing facts but is a whole concept integrating itself in the broader general views of immunophysiology and immunopathology

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VII

Delayed-type Hypersensitivity Reactions

Chairman HERMAN N EISEN MD (*St Louis, Missouri*)

Chairman s Remarks

Slowly evolving inflammatory reactions due to hypersensitivity are among the more confusing and obscure phenomena in immunology This is true mostly of course because we know so little about the basic mechanisms involved But much of the confusion in communication is unnecessary and arises from a reluctance to state questions problems and possibilities in at least semiquantitative terms

Years ago slowly evolving allergic inflammatory responses were all called delayed reactions The term delayed was used literally and innocently to describe the long interval between challenge of the sensitized host and appearance of the inflammatory response In this older view such reactions as the Arthus reaction and the serum sickness syndrome were regarded as delayed allergic reactions In recent years a changing viewpoint has led to a change in terminology so that now most of us speak not of delayed reactions but of delayed *type* reactions Emphasis is put on the qualifying term type to indicate that the central issue which confronts us in distinguishing between one and another kind of hypersensitivity response is not so much a time distinction as whether extracellular antibodies (of the type so well characterized in serum) are the essential reagents which determine the specificity of the response or whether another kind of antibody acts instead The latter substance never isolated or characterized is still widely believed by those who subscribe to its existence to be associated with cells

If we apply the term delayed in the literal sense to responses appearing 3 or 4 hours or longer after challenge then there are at least three classes of delayed allergic responses

(1) There are those reactions which like the Arthus reaction in rabbits are slow to reach maximal intensity but which are transferable with antiserums Such reactions while literally delayed would now be described by most immunologists as immediate *type* emphasis being placed on the word type to indicate that antibody in or from, serum is the specificity-determining reagent and that critical antigen antibody complexes are extracellular in origin although they probably interact with components of tissues or blood vessels or cells to initiate the inflammatory response

(2) Secondly there are slowly evolving reactions which are induced and elicited with a variety of highly purified proteins but which seem

not to be dependent on serum antibody This class of reactions first described by Dienes is referred to in the present symposium in some detail by Drs Pappenheimer Kaplan and Salvin In the earlier thorough work of Raffel and in the recent interesting studies of Uhr Salvin and Pappenheimer interdermal injection of small amounts of protein (e g, 1 μg especially in the form of specific antigen antibody precipitates made in antibody excess) leads in a short time to acquired sensitivity such that subsequent interdermal injection of the protein produces a slowly evolving inflammatory reaction Serum analyses at the time the delayed skin responses can first be elicited fail to reveal antibody by the most sensitive methods available (about 0 1 μg/ml), and serum obtained at this time fails to transfer sensitivity from an actively induced host to a normal recipient

Two issues concerning these systems must be kept in mind First in making correlations between skin reactivity and serum antibody analyses it is essential if the correlations are to be meaningful, that the specificity in both assays be directed toward the same antigenic determinant This necessity imposes severe experimental difficulties since trace contaminants are present in the most highly purified proteins now available, this problem is responsible for much of the current controversy regarding the interpretation of allergic reactions produced with some highly purified proteins The second question that must be considered in these systems is whether exceedingly low concentrations of antibody below the lowest concentration detectable by the most sensitive methods available may not be sufficient to mediate slowly evolving inflammatory responses One would expect at very low concentrations of antibody to have proportionately slow formation of antigen antibody complexes with correspondingly slow evolution of inflammatory lesions In principle, this possibility can be excluded if it is possible so to sensitize individuals that they have relatively high serum antibody concentrations but give *no* delayed inflammatory lesion on intradermal test with antigen This possibility is not ordinarily realized with protein antigens here as a rule when serum antibody concentrations rise to appreciable levels (as detected by precipitin reactions) intradermal injection of protein antigen produces inflammatory responses which appear first in to 4 hours progress and persist long enough (Arthus reaction) to make it difficult or impossible to judge whether the slower evolving response is absent In carrying out experiments of this type with proteins it is as emphasized above essential that identity of specificity between serum assay and skin response be established

(3) A third kind of delayed response is contact skin sensitivity (allergic contact dermatitis) which is induced and elicited with low molecular weight sensitizers Here the evidence for nondependence of the delayed

allergic lesion on serum antibody rests largely on observations that guinea pigs induced with sensitizer protein conjugates regularly have anaphylactic sensitivity specific for the simple determinant of the inducing conjugate but do *not* have contact skin sensitivity for the same determinant Since anaphylaxis has been demonstrated to be a function of serum antibody this situation furnishes the basis for the belief that when a guinea pig has at the same time contact skin sensitivity and anaphylactic sensitivity both responses being specific for the same determinant, the lesion of contact dermatitis is not dependent on serum antibody This situation is set forth in Table I

TABLE I

Sensitivity Induced with[a]	Anaphylactic Sensitivity — Specific for DNP[b]	Contact Skin Sensitivity — Specific for DNP
	Frequency	Frequency
DNP BGG	>90%	0%
DNFB	~50%	>90%

DNP = 2 4 dinitrophenyl DNFB = 2 4-dinitrofluorobenzene DNP BGG = 2 4-dinitrophenyl beef γ globulin induction with 0 1-0.2 μ moles dinitrophenyl in complete Freund s adjuvant

[a] Elicited with DNP on a heterologous protein (e g egg albumin or guinea pig serum proteins)

[b] Elicited with simple 2 4 -dinitrobenzene sensitizers (i.e., those whose C-1 substituents are F Cl Br SO SCl SCN etc)

The assumption underlying the foregoing argument is that specificity both in the test for anaphylaxis and in the test for contact skin sensitivity is directed toward the dinitrophenyl group In the case of anaphylaxis this seems clear beyond reasonable doubt because one induces with DNP protein A and elicits shock with a DNP conjugate made from a noncross reacting heterologous protein including for example DNP-guinea pig serum proteins In the case of contact skin sensitivity the nature of specificity has been established only indirectly and rests on the results of skin testing with a variety of simple chemicals positive reactions only being given by simple ,4 dinitrobenzenes which yield *in vivo* protein derivatives in which DNP groups are bound by covalent bonds to NH or S of amino acid residues More direct characterization of the determinant involved in contact skin sensitivity would be possible if (1) the lesion could be elicited with DNP proteins made in the test tube and/or (2) the lesion could be specifically inhibited by low molecular weight dinitrobenzenes which do not elicit by virtue of their inability to combine chemically with proteins *in vivo* Since neither of these two conditions has so far been met it must be conceded that

characterization of the determinant group involved in contact skin sensitivity rests on evidence which is only correlative in type

Setting aside the Arthus reaction which is clearly dependent on serum antibody there is a large body of experience which indicates that there do indeed exist hypersensitivity states in which specificity is determined by something other than serum antibody and which are characterized by slowly evolving inflammatory responses The fascination which the latter reactions hold for many immunologists lies in the implication that underlying such reactions is an as yet undiscovered system of antibody like substances which are cell associated and which differ in significant manner from serum antibodies But in all the experimental data which at present support this implication there exist enough ambiguities to furnish skeptics with plenty of ammunition

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*Delayed Hypersensitivity and Its Possible Relation to Antibody Formation**

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The concept that antibody molecules possess specific sites with steric configuration complementary to determinant haptophore groups on the antigen molecule has been widely accepted since it was proposed by Paul Ehrlich[19] Likewise it is generally agreed that enzyme molecules possess stereospecific binding sites complementary to their corresponding substrate molecules In bacteria enzyme formation is often specifically induced by substrate or by substances whose configuration is closely related to that of substrate Thus the ability of a mammalian cell to synthesize antibody protein with configuration complementary to determinant groupings on antigen is a property shared by even the most primitive living cells

Burnet and Fenner[4] first pointed to this similarity between induced enzyme synthesis in bacteria and antibody formation in higher animals Monod[40] has recently made a careful and critical analysis of the similarities and the differences between antibody formation and induced enzyme synthesis He has pointed out that both systems involve the *de novo* synthesis of proteins with structural sites complementary to haptophore groups on the antigen molecule or to inducer respectively Both processes involve a kind of memory effect as exemplified by the response of preinduced bacterial cells to secondary challenge with inducer and by the booster effect of a secondary challenge of the sensitized animal with specific hapten protein Recent work in the laboratories of Monod[7]

This study from the Department of Microbiology New York University College of Medicine was aided by grants from the National Institutes of Health U S Public Health Service The work was conducted in part under the sponsorship of the Commission on Immunization Armed Forces Epidemiological Board and was supported in part by the Office of the Surgeon General Department of the Army

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and of Cohn [8] has demonstrated that induced enzyme formation in bacteria occurs in two steps each of which is under separate genetic control The first of these preinduction involves formation of stereospecific permeases (presumably located on the cell membrane) which are concerned in capture of the inducer with its transport across the cell membrane and its concentration within the bacterial cell The second step involves enzyme synthesis itself

In this chapter we shall discuss evidence suggesting that antibody formation may likewise involve the induction of two complementary structures each under separate genetic control It is suggested that the first of these inducible complementary structures is that which determines the phenomena associated with the delayed hypersensitive state Certain

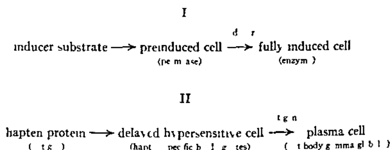


FIGURE 1 Scheme showing (I) process of induced enzyme synthesis by bacteria as a possible model for (II) antibody synthesis

of the cell types concerned in specific inflammatory reactions that characterize delayed hypersensitivity would thus correspond formalistically (as shown schematically in Figure 1) to preinduced bacteria The second inducible complementary structure would of course be the antibody gamma globulin molecule itself

The Chemical Theory of Antibody Formation

Before considering in detail the scheme shown above it seems worth while to review very briefly certain current views on antibody synthesis The theory of antibody formation as modified gamma globulin synthesis with antigen serving as template (Breinl and Haurowitz [2] Alexander [1] Mudd [4]) was expressed in its clearest and most explicit form by Pauling [46] [4] In the words of Pauling [47] The complementariness between an antibody molecule and an antigen molecule is the result of a folding of the polypeptide chain of the antibody molecule in such a way that as large a portion as possible of the chain is brought into immediate juxtaposition with a portion of the surface of the antigen molecule In addition

to similarity of surface configuration the complementariness of structure involves the juxtaposition of special combining groups such as a negatively charged group in the antibody with a positively charged group in the antigen or a hydrogen bond forming group carrying the proton with a similar group presenting an electron pair Pauling was concerned with synthesis of antibody as a protein of structure complementary to that of antigen and with the mechanism of antigen antibody interaction The theory has been criticized because it offers no explanation for the difference between a primary and a secondary antibody response it gives no satisfactory explanation of why an animal fails to make antibody against its own protein nor does it explain the phenomenon known as immunological tolerance

Persistence of Antigen in Immunized Animals

The template theory of Pauling requires that antigen must be present for continued synthesis of antibody Since low levels of circulating antibody can often be detected for years after any known antigenic stimulus then if the template theory is correct it follows that antigen must also remain for years albeit in small amounts That certain antigens can remain in hiding for many years in tissues cannot be denied Brill s disease for example has been observed as a recrudescence of typhus fever 20 years or more after the original rickettsial infection [9] Pneumococcal polysaccharide has been detected in mice one year after a single injection of 1 mg (Coons and Kaplan [11]) However protein antigens in detectable amounts have been shown to disappear rapidly from the circulation and from the tissues particularly following an immune response Thus I^{131} BSA disappears from the blood stream after intravenous injection into rabbits at an ever increasing rate (Talmadge *et al* [54]) More recent experiments by Dixon *et al* [15] on S^{35} methionine incorporation into antibody have shown a slowly declining antibody synthesis which continues long after all demonstrable antigen (I^{131} BGG in this case) has disappeared

It would be a mistake to conclude from experiments of this kind that antibody synthesis can take place in the absence of antigen It is well known that amounts of antigen far too minute to detect by any means now available suffice to produce easily measurable circulating antibody titers Thus it has been repeatedly shown that the Schick test dose (ca 0 001 μg) of diphtheria toxin is sufficient to raise the circulating antitoxin level in Schick negative human adults by 1 to 2 units or more This dose of toxin corresponds to less than 10 molecules of antigen per kilogram of body weight! If 1 μg of toxoid acted with equal efficiency a titer of 1000 to 2000 units of antitoxin per milliliter would be reached

within _ weeks after its injection corresponding to the replacement of virtually all the circulating gamma globulin by antitoxic gamma globulin (1 5–3 Gm per cent) It is obvious therefore that minute traces of protein antigens might persist undetected for long periods Koshland[32] could still detect I^{131} diphtheria toxoid in guinea pigs months after its injection even in the presence of a high titer of circulating antitoxin Garvey and Campbell[23] have observed the retention of labeled antigens for long periods by rabbit liver In the experiments of Freund[1, 22] high titers of antibody were sustained for as long as 2 years after a single dose of antigen in complete adjuvant In this case small amounts of antigen were shown to persist in depots of oil droplets containing mycobacteria and thus furnish a continuing stimulus to antibody formation over long periods of time

It is probable that *even during a secondary response*, only a small fraction of the antigen injected ever succeeds in reaching antibody forming cells Most of the antigen is catabolized and broken down in reticuloendothelial cells of the liver (Crampton and Haurowitz,[13] Coons *et al*[12]) and in other tissues including the draining lymph nodes themselves (Koshland[3]) In experiments using labeled proteins to determine the fate of antigen in animals following injection it is probable that only a very minor fraction of what is measured has anything to do with antibody formation The small amount of antigen which is actually concerned with induction of antibody synthesis may be eliminated at a far slower rate In fact it is possible that the antibody forming cells are precisely those which cannot catabolize antigen[59] In conclusion then it is our opinion that none of the available evidence is inconsistent with the basic assumption that antibodies are produced only when antigen or conceivably breakdown products containing the specific antigenic configuration are present

Antibody Formation As a Two step Process

The very fact that there exists a striking difference in response to a secondary as compared with a primary antigenic stimulus suggests that two steps are involved in the formation of antibody Some sort of priming or sensitization of certain cell types must occur as a first step in the process Primed or sensitized cells then respond differently and more efficiently to antigenic challenge than do unsensitized cells Dixon *et al*[17] described a radiosensitive initial phase preceding a radioresistant secondary phase during antibody formation in the rabbit The studies of Leduc *et al*[35] on primary and secondary antibody responses also led them to conclude that antibody formation is a process involving two steps More recent studies from Dixon s laboratory[16] have shown that lymph node cells from

donor rabbits that had already received a primary dose of antigen responded to an antigenic stimulus given after transplanting them to irradiated recipients No antibody response could be obtained in the irradiated animals that received cells taken from normal donors Similar results were obtained by Michaelides[39] who demonstrated antibody production in tissue cultures challenged with antigen *in vitro* Again only cells from donor rabbits which had previously received a primary antigenic stimulus responded to antigenic challenge given *in vitro* Cells from normal rabbits showed no demonstrable antibody response to antigen in tissue cultures Harris and Harris have obtained an antibody response to Shigella antigens in irradiated rabbits that had received lymph node cells from normal donors The objection can be raised in this case however that the donors were probably already sensitized by previous contact with the same or related organisms The reports by McKenna and Stevens[37] of primary antigenic stimulation of spleen cells *in vitro* with typhoid vaccines must be interpreted with caution since the significance of low agglutinin titers is open to question

Does the Primary Antigenic Stimulus Cause a Permanent Genetic Modification of the Cell?

The number of enzymes that can be synthesized by any given cell is probably limited Moreover immunologically as well as chemically each enzyme in the cell appears to be a distinct protein It is not surprising therefore that a different gene or group of genes seems to be concerned with the synthesis of each specific enzyme All antibodies on the other hand appear to be modifications of a single protein (or very closely related group of proteins) that is gamma globulin In man studies on the heritable disease agamma globulinemia suggest that the formation of this protein may possibly be controlled by a single gene located in the X chromosome Moreover the number of different antibodies, that is configurations of the gamma globulin molecule that a given cell can form seems to be almost unlimited since it includes antibodies against all of the species specific antigens and all of the artificial hapten configurations that man can conceivably devise

Despite considerations such as mentioned above the striking difference between the primary and secondary immune responses led Burnet and Fenner[4] to suggest that antigen induces the formation of self replicating units that continue to manufacture antibody long after complete disappearance of antigen In his more recent book Burnet[5] still insists that antibody synthesis does not require the continued presence of antigen but he has modified the theory to conform with recent findings of Dixon Coons and others cited above He now suggests that the nucleus

(but not necessarily the chromosomes themselves) is altered by antigen so as to permanently modify the type of RNA protein which carries information to the cytoplasm of the cell and its descendants It is this genocopy RNA+ which serves as template when descendants of the altered cell come into contact with antigen Burnet continues to stress the analogy to induced enzyme synthesis despite the fact that all available evidence supports the view that in bacteria enzyme synthesis ceases the moment inducer is withdrawn Schweet and Owen[51] while conceding the likelihood that antibody formation requires the presence of antigen nonetheless suggest that each new antigen actually causes a specific heritable modification of the nuclear DNA Both Burnet and Schweet and Owen cite as evidence for their views experiments of Crampton and Haurowitz[13] who demonstrated that antigen may be found soon after primary injection in nuclear fractions and of Coons *et al*,[12] who observed antigen directly within the nuclei of cells However most of the injected antigen so observed was found in cells of the reticuloendothelial system such as the Kupfer cells of the liver which apparently have little or nothing to do with antibody formation On the other hand Leduc Coons and Connelly[5] have demonstrated a veritable explosion of mitotic figures and differentiation to immature and mature plasma cells in lymph nodes following secondary stimulation with antigen The plasma cells are found in colonies As Monod[40] has pointed out and as will be discussed below such a *clonal* distribution of plasma cells does not necessarily imply that antigen has caused a directed mutation

The elegantly conceived experiments of Novick and Weiner[43] have shown that in partially induced cultures of *Escherichea coli* (using low inducer concentrations) formation of the enzyme β galactosidase is essentially an *all or none* phenomenon In other words a cell contains either its maximal complement of enzyme or none at all This inhomogeneity of partially induced cultures is explained by the existence of the permease system Once a cell has formed a threshold amount of permease it has a tremendous selective advantage over other cells which have to depend on simple diffusion for capture of inducer molecules Induced cells will retain this selective advantage for several generations after complete withdrawal of inducer since permease sites are divided among the progeny All of the daughter cells will remain induced until permease has become diluted out below its threshold concentration resulting in a clonal distribution of induced cells

It is possible that antibody formation involves an analogous primary process of induction or sensitization to form complementary surface configurations which enable certain cells to interact with and capture antigen molecules and so make antigen available to the site of gamma globulin synthesis Sensitive cells would then possess a high selective advantage

over nonsensitive cells and would retain this advantage through several generations Moreover, we must not overlook the possibility that antigen too could be divided among the daughter cells In their studies on penicillinase formation by *Bacillus cereus*, Pollock and Perret[48] showed that at most about 100 molecules of S^{35} labeled penicillin are fixed per bacterial cell This bound penicillin cannot be washed out of the cells but is diluted among the daughter cells which continue to produce enzyme for as long as the inducer remains to be divided among them These mechanisms would explain the clonal distribution of plasma cells so beautifully demonstrated by Coons in lymphoid tissue during the secondary response to antigen They would also explain Coons[10] finding that animals sensitized to ovalbumin and diphtheria toxoid and then given a secondary stimulation with both antigens show colonies of plasma cells staining by the fluorescent method either for antiovalbumin or for antitoxin but not for both Few if any cells appear capable of forming both antibodies * This observation could be explained by assuming that the first complementary antigen binding site formed on a cell will be determined by the rare chance successful collision or contact with either an ovalbumin or a toxoid molecule Thereafter that cell and its progeny gain an increasing advantage over other cells with respect to whichever antigen it first had contact with

Dubert[35] has recently carried out interesting experiments stimulated by the studies on induced enzyme synthesis Rabbits were immunized to either of two cross reacting proteins human serum albumin (HSA) or sulfanyl diazo-HSA On secondary stimulation Dubert found that it was the information gained during the primary experience with antigen that was re expressed Very similar results were obtained by Maurer and Dixon[36] using cross reacting serum proteins (albumin and globulin) from different species As Monod has pointed out[4] Dubert's observations could be explained according to the induced enzyme model provided one cell can synthesize only *one* antibody at a time The clonally distributed sensitized cells should then be exclusively specialized for the antigen towards which they were originally sensitized It will be recalled that in the original statement of his theory Pauling[46] predicted the existence of antibody molecules with combining sites directed at two distinct hapten groups In spite of all attempts both in Pauling's laboratory and those of others (Haurowitz and Schwerin[28] Eisen *et al*[29]) complementary sites directed against two haptenic groups of differing specificities have not been found on the same antibody molecule The failure to find double

* Cohn and Lennox[8] have measured bacteriophage neutralizing antibody in single isolated lymph node cells from rabbits immunized against two serologically distinct bacterial viruses Their striking experiments have shown conclusively that a small but predictable proportion of lymphoid cells do form two types of antibody molecules per cell of differing specificity

headed antibodies is in keeping with the evidence which suggests that ordinarily a single cell produces only one specific antibody

Induction of the Delayed Hypersensitive State

It has been known for a long time that under certain conditions antigens can induce specific and long lasting changes in host cell reactivities which, despite their immunological specificity do not appear to depend on the formation of the conventional type of serum antibodies In suitably sensitized guinea pigs or human subjects intradermal challenge with minute amounts of antigen will initiate characteristic progressive inflammatory lesions even when the most sensitive tests fail to detect the presence of antibody in the circulation Such skin lesions are indicative of the delayed hypersensitive state Experimental study of the delayed hypersensitive state has been rendered difficult until recently, because the methods used to sensitize to a given antigen almost invariably caused simultaneous formation of serum antibody *

It has recently been shown[56] that delayed sensitivity can be induced in guinea pigs and in man to single protein antigens by one intradermal injection of a small amount of the antigen (1 μg or less) in the form of its complex with excess antibody Under these conditions circulating antibody formation is suppressed for 2 or more weeks in guinea pigs In such animals it is possible to study delayed hypersensitivity reactions to a given antigen uncomplicated with reactions triggered by interaction of the same antigen with conventional serum antibody That formation of the factor (or factors) responsible for delayed sensitivity is under separate genetic control from gamma globulin synthesis is shown by the ease with which patients with congenital agamma globulinemia † can be sensitized to antigens against which they cannot produce conventional antibody

In man the delayed hypersensitive state can be transferred to normal recipients not only by intact cells of the lymphocyte series taken from the peripheral blood of highly sensitive donors but also by DNase treated extracts from these cells[33] Moreover there is some recent evidence which suggests that when the sensitive donor s cells are allowed to interact *in vitro* with antigen the specific factor involved in the cell

* Sensitization to certain reactive chemicals by topical application to the skin often fails to induce demonstrable circulating antibody and thus furnishes an exception to this statement

† It has recently become fashionable to speak of this disease as *hypo* gamma globulinemia because small amounts of protein are precipitated from patients serum by antisera supposedly specific for human gamma globulin It is questionable however whether the small amounts of gamma globulin found by the immunochemical method have anything to do with antibody gamma globulin because of the universal failure to detect circulating antibody in these patients by the most sensitive tests

transfer of sensitivity may be released into the supernate [54] From experiments on desensitization of guinea pigs sensitized to more than one antigen it would appear that the interaction between sensitive cells and antigen is as specific as is the interaction between serum antibody and antigen [55] Since the delayed sensitive state usually persists (in man at least) for many years even in the absence of known contact with antigen and since the cell types implicated (i e cells of lymphocyte series) are relatively short lived it seems likely that the transfer reaction is continually taking place within the actively sensitized individual In this connection the recent demonstration by Hamilton [9] of prolonged retention of C^{14} labeled purines by lymphocytes has suggested the interesting possibility of reutilization of large fragments of DNA RNA and possibly nucleoprotein

Delayed Sensitivity and Antibody Formation

If induction of the delayed hypersensitive state is the first step in the antibody forming process as suggested in Figure 1 then stimulation of sensitized animals with free antigen should cause a secondary type of antibody response This would be similar to the increased enzyme synthesis of preinduced bacteria following a fresh stimulation with low levels of inducer Experiments such as described below have shown that sensitized guinea pigs do react to challenge with antigen by showing rapid and vigorous antibody production

Guinea pigs respond rather poorly to intravenous injection of certain antigens such as bovine serum albumin (BSA) Curve A of Figure 2 shows the now familiar pattern of elimination of I^{131} labeled BSA from the circulation of control guinea pigs sensitized to diphtheria toxoid after receiving 1 mg I^{131} BSA intravenously In general most of the animals after equilibration with the tissues show a half life of BSA in the circulation of close to 2 days (Curve A Figure 2) In agreement with Weigle and Dixon [58] and others we have never been able to demonstrate anti BSA antibody in the serum of any of these animals up to at least 4 weeks after a primary intravenous injection of 1 mg I^{131} BSA

When guinea pigs are given a single intradermal injection of 3 μg or less of BSA in the form of a washed specific precipitate formed in excess rabbit antibody and suspended in oil water emulsion (incomplete adjuvant) they develop sensitivity and show delayed skin reactions if tested with BSA after the fourth day If the sensitized animals remain *untested* no circulating anti BSA can be detected for at least 3 to 4 weeks even by the sensitive passive cutaneous anaphylaxis (PCA) test [44]

If however sensitized animals are challenged intravenously with I^{131} BSA circulating antibody can be detected within a few days

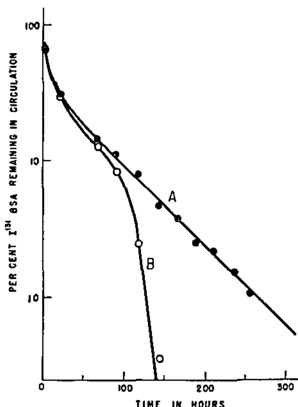


FIGURE 2 Elimination of I131 BSA from circulation of sensitized guinea pigs All animals challenged intravenously with 2 5 mg I131 BSA per kilogram (4 × 10⁶ counts per milligram) Curve A 3 animals sensitized with 2 5 µg diphtheria toxoid as toxoid antitoxin complex 8 days before challenge Curve B 5 animals sensitized with 3 µg BSA as BSA anti BSA complex 8 days before challenge

Curve B of Figure shows the antigen elimination curve of 5 guinea pigs sensitized 8 days previously with an immune complex containing 3 µg BSA Following intravenous challenge with 1 mg I131 BSA elimination of the circulating antigen occurred at a faster rate (owing to anti BSA formation) than in the toxoid sensitive control animals and became apparent within 7 to 96 hours Elimination of antigen from the blood was complete by the fifth or sixth day Twenty four hours later excess circulating anti BSA could be detected by PCA test In Figure 3 we have plotted against time the ratio of the I131 BSA which remained in the circulation of the BSA sensitive animals to that which remained in the toxoid sensitive control animals at intervals following intravenous challenge

Figure 3 shows that when the sensitizing dose of immune precipitate containing 3 µg BSA was given 8 days before intravenous challenge with

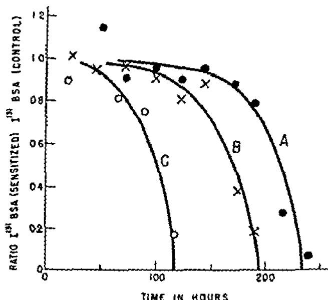


FIGURE 3 Effect of interval between sensitizing and challenge dose on elimination of I^{131} PSA from circulation of sensitized guinea pigs Sensitizing dose 3 μg BSA as BSA anti BSA complex Challenge dose 25 mg I^{131} BSA per kilogram Control animals sensitized to diphtheria toxoid Curve A sensitized and challenged simultaneously Curve B sensitized 3 days before intravenous challenge Curve C sensitized 8 days before intravenous challenge

1 mg I^{131} BSA the latter (on the average) disappeared from the circulation within about 130 hours (5 4 days) when given 3 days previously elimination required 9 days and when given simultaneously 10 4 days An increased rate of elimination of antigen from the circulation could be detected within 4 6 and 7 days respectively after intravenous BSA Thus even a tiny intradermal sensitizing dose of specific immune complex caused a profound alteration in the animals ability to form antibody after intravenous challenge with free antigen While other interpretations are possible the results are at least consistent with the hypothesis that delayed sensitization is the primary step in antibody formation

ANTIBODY RESPONSE IN ANIMALS SENSITIZED BY CELL TRANSFER

The temptation to link delayed hypersensitivity to the process of antibody formation is a great one because it is ordinarily difficult if not impossible to separate experimentally the two kinds of response to antigenic stimulation There is good evidence (far more obvious in some animal species e g the guinea pig than in others) that in any method of immunization antibody formation is *always* accompanied by some

degree of delayed sensitization Conversely, delayed sensitization is always accompanied by antibody formation except under special circumstances such as in the agamma globulinemic individual Chase has pointed out that cells from the same tissues are concerned with transfer of delayed sensitivity as are concerned with antibody formation These cells are present in lymphoid tissue spleen, and in various types of exudates Wager and Chase[57] found that when spleen and lymph node cells from rabbits that had received a rather large dose of diphtheria toxoid a few days previously were transferred to normal rabbits a biphasic curve of antitoxin formation took place in the recipients The initial antitoxin formed which could be detected within 4 to 48 hours was almost certainly produced by the donor cells since this initial phase of antibody formation was eliminated if the cells were killed by heating or treatment with chloroform prior to transfer The second and later phase was clearly due to transfer of antigen and to an active primary antitoxin response by the recipient This second phase could be prevented by reducing the dose of toxoid given to the donor rabbits Recently Chase[6] has shown that transfer of delayed sensitivity in guinea pigs by similar cells will also give a biphasic response in many of the recipient animals In transfer of sensitivity to picryl chloride for example recipients frequently show a transient delayed skin reactivity that diminishes 3 to 4 days after transfer only to be followed a few days later by a second rise in sensitivity which is of long duration By analogy with the experiments of Wager and Chase[5] it seems likely that the initial phase is due to interaction of the challenge dose of picryl chloride (or its protein conjugate) with the transferred donor cells There is very little evidence to indicate that transfer factor * is transferred from the donor to recipient cells in the guinea pig as has been suggested to explain transfer of delayed sensitivity from man to man[45] Metaxas and Metaxas Buehler[38] arrived at a similar conclusion as a result of their observations on the bimodal response of recipient guinea pigs sensitized to tuberculin by cell transfer In Chase's experiments treatment of the donor cells with chloroform prevented the appearance of *both* phases of sensitivity in the recipient animals However this fact certainly does not rule out the possibility or even the likelihood that the secondary and more permanent phase of sensitivity was *actively* induced in the recipients by traces of antigen (in this case presumably picrylated protein) carried over with the donor cells For example antibody formed by the transferred donor cells might interact with small

* The term transfer factor has been used to designate the specific cellular factor concerned in delayed inflammatory reactions[45] It has been suggested that it is this same factor in extracts of human leukocytes which is responsible for transfer of delayed sensitivity from man to man With one possible exception[80] all attempts to transfer delayed sensitivity in laboratory animals with nonliving cell preparations have remained unsubstantiated

amounts of transferred antigen to bring about active sensitization of the recipient animals

There have been a good many recent reports of antibody formation following secondary antigenic stimulation both *in vitro* in tissue culture and *in vivo* in irradiated and unirradiated animals using cells taken from animals that had received a primary stimulation of antigen some time previously Thus Dixon *et al*[16] and Stavitsky[63] have demonstrated a secondary response in irradiated and in normal rabbits that had received lymph node peritoneal exudate or splenic cells from donor rabbits given a primary stimulation with BSA BGG or diphtheria toxoid some weeks prior to transfer Harris and Harris[7] have measured the appearance of antiparadysentery agglutinins in recipients of donor cells from normal rabbits challenged *in vitro* with killed organisms or with soluble extracts * In all of these cases the evidence suggests that it is only the *donor* cells which respond to the antigen and that the recipient animal plays the role of a culture medium for proliferation and differentiation of the primed donor cells Since delayed hypersensitivity was also induced by the primary stimulation with antigen sensitized cells must have been among those transferred Thus we return to the question of prime importance Is it the delayed sensitive cell that proliferates and differentiates to an antibody forming cell on stimulation with antigen?

Desensitization

The early appearance of delayed hypersensitivity in animals immunized with proteins incorporated in adjuvants containing tubercle bacilli led Dienes and Schoenheit[14] to suggest that delayed sensitivity might represent an early stage in antibody formation Their viewpoints soon found support from the observations of Mote and Jones[41] and of Simon and Rackemann[52] who showed that in humans immunized intracutaneously with small amounts of foreign serum proteins delayed skin reactivity appeared several days before reactivity of the immediate type associated with circulating antibody Similar observations have recently been made in guinea pigs by Salvin[50] following intracutaneous immunization with minute doses of ovalbumin and of diphtheria toxoid Dienes and Schoenheit's suggestion seems to have been overlooked or disregarded in most theories of antibody formation probably because the reactions of delayed hypersensitivity are rarely a conspicuous or obvious feature during the ordinary course of immunization

* Because of the close antigenic relationship of the vaccines used by the Harrises to the normal flora of the rabbit intestine it seems likely that they were observing a secondary antibody response in their recipient animals

The failure of most workers to observe reactions indicative of the delayed hypersensitive state during the course of immunization of animals or of man may be accounted for in several ways

1 Delayed reactions are most prominent when antigen is administered intracutaneously Unless animals are unusually sensitive reactions will not be conspicuous following subcutaneous or intramuscular injection of antigen

2 During ordinary immunization procedures, booster injections may give rise to an Arthus type of inflammation An active Arthus reaction is probably the *combined* result of inflammation due to interaction of antigen and circulating antibody with that due to delayed sensitivity (Gell and Hinde) As Gell [4] points out the *delayed* component of the active Arthus reaction has been overlooked until very recently

3 With the relatively high doses of antigen customarily used in immunization *desensitization* occurs which often reduces or even prevents entirely the appearance of specific delayed inflammatory reactions

Except for attempts to desensitize tuberculous guinea pigs (or man) to tuberculin – attempts that were frequently unsuccessful – there have been almost no studies on desensitization of the delayed hypersensitive state Recent studies in our laboratory have shown that guinea pigs rendered exquisitely delayed sensitive to single protein antigens can be completely and specifically desensitized by one injection of 0 5 to 1 mg of the antigen so that they no longer show delayed skin reactions [5] Moreover the macroscopic appearance of the specific delayed skin lesions can be completely prevented even when the desensitizing dose of antigen is given as late as hours *after* skin test It is not surprising to find therefore that guinea pigs sensitive to ovalbumin (Ea) show no visible reactions when skin tested with 1 mg of this antigen while in other animals sensitized in the identical manner one millionth this quantity of Ea 0 001 μg will cause delayed skin lesions measuring 0 mm in diameter when seen at 4 hours and 1 to 10 μg results in lesions 50 to 60 mm in diameter Since most routine immunization procedures involve booster injections containing milligram quantities of antigen delayed skin reactions may not be observed even when the animals are actually sensitive

From the cytological evidence it would seem most unlikely that more than a few of the sensitized cells which participate in the specific delayed inflammatory process actually proliferate and differentiate into antibody forming plasma cells on stimulation with antigen The desensitizing dose of antigen which also acts as a secondary stimulus in immunization must interrupt the local inflammatory process by blocking specific cell sites so as to prevent their further interaction with antigen We may suppose that in those cells destined to form antibody the specific interaction with antigen results in its retention to alter specifically gamma globulin synthesis

Conclusions

The capacity to form proteins of configuration complementary to inducer molecules appears to be an adaptive mechanism that is common to most if not to all living cells. Looked upon in this broad sense the comparison between induced bacterial enzyme synthesis and antibody formation in animal cells is probably a meaningful one. In both cases we are concerned with the *de novo* synthesis of proteins of structure complementary to that of the inducer, whether substrate on the one hand or haptenic groups on the other.

There is a growing body of evidence to support the view that injection of antigen into an animal induces synthesis of *two* types of structures with configurations complementary to determinant groupings on the antigen molecule. One of these antigen binding sites is fixed to cells and is responsible for the reactivity associated with the delayed hypersensitive state. The other is antibody gamma globulin. The ease with which patients with the heritable disease agammaglobulinemia can be sensitized to protein antigens indicates that the synthesis of each structure must be under separate genetic control. In both cases antigen is probably required as a template to direct the folding of polypeptide chains to complementary configurations (Pauling[49] Karush). In this chapter we have been concerned with how the formation of each of these two antigen induced complementary structures is related to the other. We are proposing that they represent successive steps in antibody synthesis. It is suggested that the primary antigen induced complementary configuration is located at or near the surface of cells and is the specific factor which accounts for the inflammatory reactions characteristic of the delayed hypersensitive state. Secondary stimulation of sensitized animals with small doses of antigen may provoke a delayed progressive inflammatory reaction or larger doses of antigen may result in desensitization. In either case specific interaction of antigen with the complementary binding sites of *certain* sensitized cells results in the capture of antigen by the cell and its retention at the site of gamma globulin synthesis. There is no need to postulate that the transport of antigen to the site of gamma globulin synthesis involves enzymatic action analogous to bacterial permease. Such pre plasma cells produce antibody gamma globulin and differentiate into plasma cells. A clonal distribution of antibody forming plasma cells will result from secondary antigenic stimulation if on proliferation of sensitized cells the antigen induced binding sites are divided so that the daughter cells remain hypersensitive (in analogy to the progeny of pre induced bacteria).

The above theory has the merit of explaining the difference between the primary and secondary responses to antigenic stimulation without

assuming that each antigen is capable of causing something akin to a specific directed mutation (Burnet[3] Schweet and Owen[51]) Nor is it necessary to assume that the antigen selects its own complementary configuration from among an unlimited number of pre existing errors" in normal gamma globulin synthesis as has been suggested by Jerne[29]

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*The Cellular Response in Forms of Delayed- and Immediate-Type Skin Reactions in the Guinea Pig**

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In the early 1930's Dienes and collaborators [*] published several reports dealing with a feature of the hypersensitivity response of animals to injection of foreign protein only dimly recognized previously—namely, a phase of delayed type skin hypersensitivity early in the course of sensitization before the usual development of immediate or Arthus type sensitivity. This delayed type skin reactivity usually appeared on the fourth or fifth day after injection of protein antigen. It was quite marked in tuberculous guinea pigs; however, it was a definite feature of the reactivity of normal animals as well. Such a skin reaction elicited in a normal guinea pig on the sixth day after an intraperitoneal injection of egg white or horse serum showed the following general characteristics. After 3 hours usually no visible reaction was present although rarely a faint erythematous spot might be made out; at 6 hours a small zone of erythema usually no more than 10 to 15 mm in diameter was apparent with evidence of slight induration. The reaction was increased slightly in size at 24 hours in most instances persisting for 24 to 48 hours. In the more sensitive animals a small zone of blanching could be observed at 24 hours in the center of the erythematous indurated reaction site. In its timing and gross appearance this early phase reaction thus resembled a tuberculin test of mild intensity. Furthermore, like the tuberculin reaction it could not be elicited following passive transfer of serum. In addition, animals injected intravenously with antigen during this early sensitizing phase were not susceptible to anaphylaxis. Dienes and Mallory[5] carried out a study of the comparative histopathology of this early phase reaction and the mild tuberculin reaction and came to the conclusion that the cellular responses characteristic of these two reactions possessed certain features

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in common – in particular an early infiltration of mononuclear cells and their accumulation about small vessels and nerves and a relative numerical predominance of mononuclear cells over polymorphonuclear cells These histologic studies suggested a close relationship between these two varieties of delayed type hypersensitivity reaction At the time of these studies passive transfer of delayed type hypersensitivity by cells had not as yet been published by Landsteiner and Chase,[1] [11] and cell transfer experiments had not been attempted subsequently

The early phase of delayed skin reaction to protein was quite transient, and was superseded about the seventh or eighth day by the classical immediate type erythematous wheal Despite the dissimilarity in the gross appearance of these two types of reaction histologic study suggested a possible interrelationship It was noted by Dienes and Mallory that this immediate type reaction was characterized not only by marked edema and a profuse infiltration of polymorphonuclear cells but also by focal accumulations of mononuclear cells particularly about vessels and nerves Such a mononuclear cell response was not noted in the immediate type reaction elicited following *passive* transfer with serum Because this mononuclear reaction appeared only in *actively* sensitized animals, and because it resembled the histology of the early phase delayed reaction these authors suggested that it might represent the cellular aspect of a delayed component which was carried over into the phase of immediate or Arthus type reactivity

This special significance given to the relative predominance of mononuclear cells in the tuberculin reaction has been disputed by Rich[13] who found an initial predominance of polymorphonuclear cells in the exudate (cf also Follis[7]) and concluded that the succession of events in the tuberculin reaction was that characteristic of ordinary acute inflammation Rich however agreed that mononuclear cells became predominant in a tuberculin reaction earlier than in the inflammatory reaction caused by most irritants in a nontuberculous body Laporte[12] had noted that a mononuclear predominance was especially marked in mild tuberculin reactions More recently Gell and Hinde[8] concluded that the tuberculin reaction differs in characteristic pattern from nonspecific acute inflammation and that its essential feature is the infiltration and proliferation of mononuclear cells although infiltration of polymorphonuclears was a transient response in the early stages of the lesion These authors also supported the concept that the Arthus reaction was composite in nature and included a delayed mononuclear component[9]

Factors perhaps responsible for the difference in the cellular responses in the tuberculin reaction noted by various authors include the relative intensity of the lesion examined the time of examination in relationship to the development of the reaction and the degree of sensitivity of the

animal at time of skin test It seems probable that the more characteristic, or possibly primary, cell response in the tuberculin reaction may be masked by secondary inflammatory factors arising as a result of tissue injury in the absence of detectable necrosis It is essential for such studies that mild reactions be examined preferably in the early stages of their development and in guinea pigs showing a moderate rather than a strong degree of hypersensitiveness [8]

In 1951 these early experiments of Dienes and collaborators were repeated in connection with other work [10] The results to be reported at this time are concerned essentially with the nature of the early cellular response in various forms of dermal hypersensitivity in the guinea pig insofar as this could be determined from study of biopsies of the skin lesion Because the cellular reaction was noted to vary to some extent both qualitatively and quantitatively in the different layers of the skin attention was given especially to the cell population appearing in the highly vascular loose connective tissue layer of the lower dermis just above the musculofascial layer

Materials and Methods

Sensitization and Skin Tests

Male albino pigs weighing between 350 and 450 Gm were used throughout this work Three milligrams of a solution of fresh egg white protein in physiologic saline was injected subcutaneously in the left inguinal region and these animals later were tested for either early phase delayed or Arthus type reactivity Skin tests were carried out according to the following schedule

For the early phase delayed reaction the animals were skin tested on the fourth and fifth days with 0.05 mg of protein At 6 and 24 hours after the skin injections the reactions in the skin were measured as to size degree of swelling or induration and degree of erythema Skin biopsies of 6 hour and 24 hour reactions were excised while the animals were under Nembutal anesthesia and the animals were then sacrificed

For the immediate type reaction the animals were skin tested on the tenth and eleventh days with 0.05 mg of egg white and 6 hour and 24 hour reactions were biopsied as described above

In certain cases after biopsy of the early phase reaction the same animals were later tested for the immediate type reaction at 10 days and these lesions also were biopsied

The immediate type reaction was also examined in animals following passive transfer of serum Antiserum was prepared in 500 Gm guinea pigs following injection with egg white in Freund's complete adjuvant and

normal guinea pigs were passively sensitized by injection of 5 to 10 ml of serum intravenously or intraperitoneally Twelve to 4 hours later the animals were skin tested with 0 10 mg of egg white and skin sites corresponding to 6 and 4 hour reactions were biopsied

In the case of tuberculin hypersensitivity, the animals were injected with 10 mg of BCG organisms into the testis After 7 days they were skin tested with a 1 50 dilution of old tuberculin and biopsies were taken of the skin reactions at 6 and 4 hours

The skin sections were prepared for study as outlined by Dienes and Mallory The area of the skin reaction was marked out with India ink and the whole ring of tissue excised using a curved scissors The skin was mounted on corks with glass pins and fixation was carried out in Zenker acetic fluid Histologic sections were cut through the center of the skin reaction and mm on both sides and were finally stained with hematoxylin and eosin

Normal control guinea pigs were also injected intradermally with the test antigens and the sites of injection excised at intervals as in the case of the experimental animals

Results

The results obtained with skin tests carried out on the fourth day after the subcutaneous injection of 3 0 mg of egg white are shown in Table I At 6 hours a mildly erythematous slightly indurated lesion could gen

TABLE I Early phase Delayed Reaction to Egg White

Guinea Pig Number	6 hour Reaction †	24 hour Reaction
9	1? × 16 ++ R	10 × 13 ± P
?9	10 × 12 ± P	12 × 13 ± R
35	15 × 15 ++ R	11 × 1? + R
38	10 × 10 + P	6 × 8 ± P
105	9 × 10 ± P	8 × 9 ± P
107	8 × 9 ± P	11 × 11 + P
11?	10 × 11 + P	15 × 19 ++ R
1?9	1? × 14 + P	10 × 10 + P
133	18 × 19 + P	10 × 10 ± P
136	?0 × ?0 ± P	Negative
148	8 × 8 ++ R	18 × 19 ++ R
149	9 × 9 + P	18 × 18 + P

Animals injected subcutaneously with 3 0 mg egg white protein and skin tested 4 days later with a dose of 0 05 mg

† Skin readings expressed in terms of size of diameters in millimeters relative skin thickness graded arbitrarily from 0 to 4+ and degree of erythema (P = pink R = red)

erally be observed There was some variability as to the time course of the reaction In some animals the size induration and degree of erythema diminished slightly at 4 hours in others there was a marked intensification of the reaction with firm induration and occasionally with a zone of central blanching

These characteristics of the early phase delayed reaction are to be contrasted with the immediate type reactions observed at 10 days as shown in Table II Here the maximal reaction occurred between and

TABLE II IMMEDIATE TYPE REACTION TO EGG WHITE

Guinea Pig Number	2 hour Reaction	6 hour Reaction	24 hour Reaction
9	20 × 25 +++ P	25 × 28 +++ P	Negative
29	25 × 30 +++ P	30 × 32 +++ P	18 × 20 ++ P
35	20 × 22 ++++ P	25 × 26 ++++ R	12 × 12 + P
38	20 × 22 +++ P	30 × 35 ++++ R	Negative
24	20 × 25 ++ P	28 × 28 +++ R	Negative
26	30 × 30 +++ R	30 × 33 +++ R	23 × 25 + P
44	32 × 35 ++ P	35 × 33 ++ R	23 × 25 + P
49	21 × 22 +++ R	25 × 25 ++++ R	Diffuse ++ P
50	20 × 22 ++ P	20 × 22 + R	Negative
60	30 × 30 +++ P	35 × 35 +++ R	Diffuse ++ P

Animals injected subcutaneously with 3 0 mg egg white protein and skin tested 10 days later with a dose of 0 05 mg

6 hours characterized grossly by marked erythema and a boggy edematous swelling and showed a subsequent rapid decline At 4 hours usually there was little or no visible reaction however a large diffuse area of edematous thickening of the skin could still be felt No visible hemorrhage or necrosis was produced in these reactions

The tuberculin reactions studied are presented in Table III It will be noted that most of the animals gave mild positive reactions at 6 hours with a characteristic increase in size erythema and induration at 4 hours

The histologic features of these skin reactions may be next illustrated Figures 1 and 2 present the typical appearance of the early phase delayed skin reaction at 6 hours There is a moderate infiltration of cells largely mononuclear in type dispersed in the loose connective tissue between the corium and muscular layer as well as proliferation of mononuclear elements collected focally about arterioles veins and nerves Polymorphonuclear neutrophiles and eosinophiles usually numbered less than a third of the total cellular infiltrate The vessels were dilated however edema was present only to a mild degree In the dense connective tissue

TABLE III Tuberculin Reaction *

Guinea Pig Number	6 hour Reaction	24 hour Reaction
15	11 × 11 + P	9 × 10 + R
16	7 × 8 ± P	10 × 10 + R
20	15 × 17 ++ R	11 × 12 ++ R
30	11 × 13 + R	14 × 16 ++ R
40	15 × 17 + R	15 × 18 ++ R
46	12 × 12 + R	16 × 18 ++ R
47	8 × 9 ± P	10 × 14 + R
48	Negative	15 × 15 ++ P
52	10 × 10 + R	18 × 18 +++ R
53	8 × 10 ± P	15 × 15 ++ P
58	11 × 11 + P	15 × 17 ++ R
61	9 × 10 + R	14 × 15 ++ R

Animals injected with 10 mg tubercle bacilli (BCG) into the testis and skin tested 7 or 10 days later with 1/50 O T Animals 15 16 20 and 30 tested at 7 days, remainder at 10 days

of the subepidermal region a marked increase in cellularity of mesenchymal elements was also to be observed

Differential cell counts of representative sections at 6 hours are given in Table IV in evidence of the mononuclear predominance of the cellular reaction

At 4 hours as shown in Figures 3 and 4 a marked increase in the number of infiltrating and proliferating mononuclear cells was apparent throughout the whole section while the polymorphonuclear cells became scanty

TABLE IV Cellular Response of Delayed type Reactions at 6 Hours

Hypersensitivity Reaction	Guinea Pig Number	Gross Reaction	Differential Cell Counts % Monos	% Polys
Early phase reaction to egg white	9	12 × 16 ++ P	70	30
	29	10 × 12 ± P	67	33
	35	15 × 15 ++ R	76	24
	38	10 × 10 + P	56	44
Tuberculin reaction	15	11 × 11 + P	75	25
	16	7 × 8 ± P	48	52
	20	15 × 17 ++ R	33	67
	30	11 × 13 + R	48	52

Based on differential count of at least 400 cells in areolar portion of lower dermis

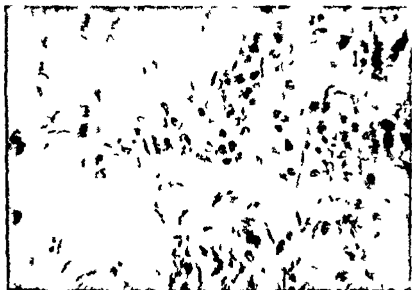


Figure 1



Figure 2

Early phase delayed reaction to egg white at 6 hours The cellular response is predominantly mononuclear with few scattered neutrophiles and eosinophiles Mononuclear cells accumulate typically in focal collections about small vessels



Figure 3



Figure 4

Early phase delayed reaction 24 hours Focal proliferation and infiltration by mononuclear cells Polymorphonuclears are rare

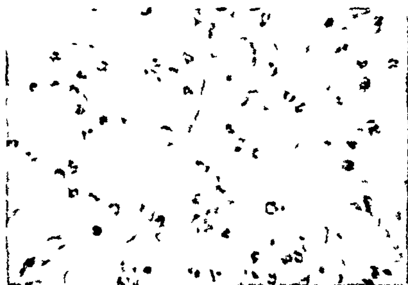


FIGURE 5 Passive Arthus reaction at 2 hours Infiltration by polymorphonuclear neutrophiles and eosinophiles in edema fluid Mononuclear cells rare

In contrast with this early phase delayed reaction is the Arthus reaction which is illustrated first in its typical appearance in animals *passively* sensitized with antiserum (Figure 5) The section is that of a skin reaction at hours It will be noted that the cellular response is exclusively that of polymorphonuclear cells including both neutrophiles and eosinophiles thickly scattered throughout the edema fluid Focal collections of mononuclear cells were entirely absent although occasionally scattered single mononuclear cells were noted A similar picture was seen in the 6-hour section with perhaps an increase in scattered mononuclear elements Focal collections were not noted nor was there evidence of a proliferative response of mesenchymal elements in the subepidermal region

The comparable appearance of the Arthus type reaction at 6 hours in *actively* sensitized animals is shown in Figures 6 and 7 Again the thickly scattered neutrophilic and eosinophilic polymorphonuclear cells may be observed throughout the edema fluid However infiltration and proliferation *of mononuclear cells also constitute a significant component of the* cellular reaction The accumulation of these cells about small vessels and nerves as well as the increase in mesenchymal elements in the subepidermal area resembles the picture of the early phase delayed response

The histology of a mild tuberculin reaction at 6 hours representative of those observed in the present study is illustrated in Figures 8 9 and 10

At 6 hours there is a moderate infiltration by both polymorphonuclear and mononuclear cells and multifocal proliferation of mesenchymal cells in the loose areolar tissue as well as in the subepidermal region. Eosinophiles are absent or rare. The relative proportion of polymorphonuclear cells is quite variable, however, in the mildest lesions it is somewhat less than the mononuclear elements. Representative cell counts in the tuberculin reaction at 6 hours are given in Table IV.

Thus in both the early phase reaction and the mild tuberculin reaction there occurred a substantial infiltration and proliferation of mononuclear cells in the loose areolar as well as the compact connective tissue layers of the skin and a somewhat lesser polymorphonuclear response, although this latter was variable in the tuberculin reaction. Further, in both the early phase reaction and the tuberculin reaction the relative absence of edema and eosinophiles was a fairly constant characteristic.

Recent work by Uhr, Salvin and Pappenheimer[15] and by Salvin[14] has indicated that delayed type reactions to protein antigens may follow their injection intradermally in the form of antigen antibody precipitate or as an emulsion in incomplete Freund's adjuvant. This delayed hypersensitivity may be passively transferred with lymph node cells. Salvin[14] has pointed out that the delayed type reactivity following sensitization with ovalbumin plus incomplete adjuvant was phasic in nature in that after persisting for some weeks it was superseded by Arthus type reactivity. This phase of delayed type reactivity following injection of protein seemed comparable except with respect to its duration to the early phase delayed reaction to egg white described in this work. The essential similarity of these two forms of delayed hypersensitivity to protein was confirmed. Guinea pigs injected intradermally with crystalline egg albumin in incomplete Freund's adjuvant as described by Salvin gave typical delayed type reactions histologically. Figures 11 and 12 show a representative reaction in a guinea pig sensitized with 3 μg of crystalline ovalbumin and skin tested on the sixth day with 3 μg of antigen. The size of this skin reaction at 6 hours was 9 by 11 mm, with 1+ induration and 1+ erythema, at 24 hours 13 by 14 mm with ±+ induration and + erythema. At 6 hours the sections showed a mild mononuclear proliferative response both in areolar tissue and in the subepidermal region, with only a few polymorphonuclear cells to be observed. At 24 hours the mononuclear response was remarkably increased in all layers of the skin. Edema and eosinophile cell infiltration were essentially absent. The histologic picture was thus quite similar to the early phase reaction to egg white.

In summary, the present work has confirmed the previous report of Dienes and Mallory that skin sensitivity of a delayed type appears briefly in the early phase of sensitization to foreign protein yielding to a later

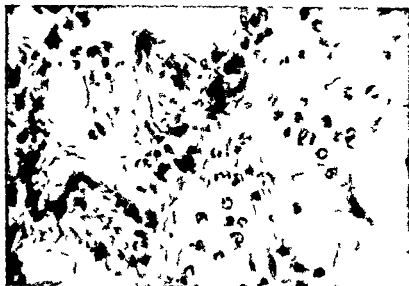


FIGURE 6

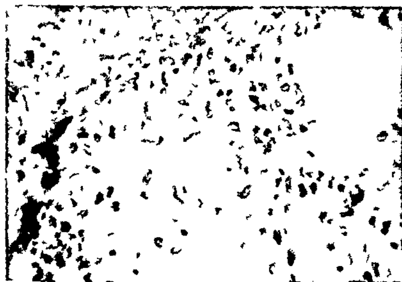


FIGURE 7

Active Arthus reaction at 6 hours Infiltration by neutrophiles and eosinophiles and focal collections of mononuclear cells

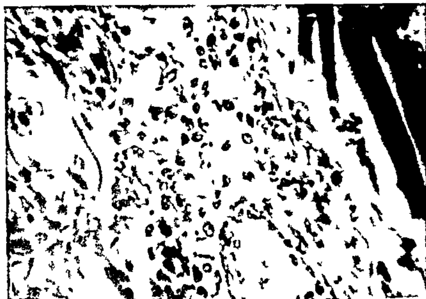


Figure 8

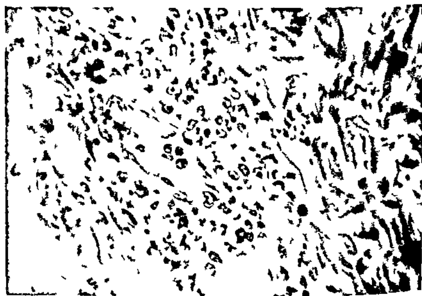


Figure 9

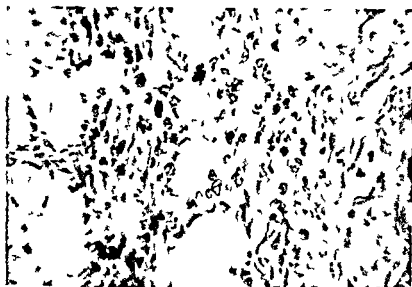


FIGURE 10

Tuberculin reaction at 6 hours There is a substantial proliferative and infiltrative response of mononuclear elements however a significant infiltration of polymorphonuclear cells is also evident in these three sections

phase of anaphylactic reactivity This early phase delayed reaction resembled the mild tuberculin reaction in certain of its histologic features notably the marked mononuclear cell infiltration and proliferation The immediate type or Arthus reaction in actively sensitized animals exhibited a mononuclear component in its characteristic cellular response suggesting the presence of a delayed component in its development The basis for the association of mononuclear cell infiltration and proliferation with delayed hypersensitivity reactions is unknown One possible explanation is that mononuclear cells are characterized by a state of responsiveness to specific antigen which results from their sensitized condition This view is supported by cell transfer studies which indicate that the transfer factor is associated with cells of the mononuclear series However the cellular response in the tissues may be subject to other complex factors as yet not understood The further question whether the underlying mechanism of delayed type hypersensitivity to protein antigen is truly *identical* with that of hypersensitivity to tuberculin cannot of course be answered by morphologic studies The available information however does point to their basic similarity

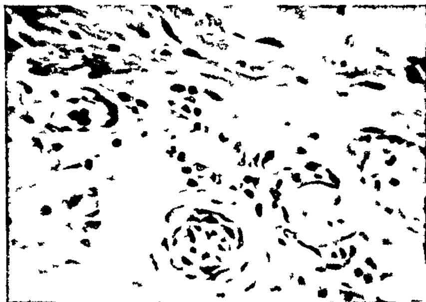


FIGURE 11



FIGURE 1

Delayed reaction to crystalline ovalbumin following intradermal sensitization with ovalbumin in incomplete Freund's adjuvant. The cellular response at 6 hours (Figure 11) and at 4 hours (Figure 1) is almost exclusively mono nuclear

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DESIGNATED DISCUSSION

SAMUEL B SALVIN (Hamilton Montana) Delayed type hypersensitivity to a soluble highly purified protein can precede Arthus type hypersensitivity and production of circulating antibody [4] This order in the development of hypersensitivity in guinea pigs has been indicated by both histologic and immunologic methods [1 3 4] With the administration of a large dose of protein antigen the sensitization process passes hastily through the stage of delayed hypersensitivity to the production of the conventional type of circulating antibody and the concurrent development of Arthus type hypersensitivity With the antigen administered in minute quantities via the intracutaneous route, sensitization proceeds less rapidly so that the delayed hypersensitivity is more pronounced develops more slowly and lasts for a longer period Eventually however, depending on the nature and dose of the sensitizing antigen circulating antibody and an Arthus type hypersensitivity usually develop Ovalbumin and diphtheria toxoid in amounts as low as 0 03 μg have produced delayed type hypersensitivity in 450 Gm guinea pigs of the Hartley strain

This delayed hypersensitivity that follows the intracutaneous injection of minute quantities of soluble protein antigen is typical The reaction is not grossly observable until 6 or 8 hours after intracutaneous testing reaches its maximum at about 24 hours and then subsides in intensity With the diphtheria toxoid antitoxin system no circulating antibody can be detected by the rabbit intracutaneous test Thus less than 0 001 unit of antitoxin or 0 00 5 μg of antibody nitrogen if any is in circulation Transfer of serum to a recipient animal does not produce hypersensitivity of any kind Transfer of cells from lymph nodes or from peritoneal fluid to recipient animals produces delayed hypersensitivity Histologic examination of the skin testing site reveals infiltration of mononuclear cells

Skin testing of the guinea pigs during the first 4 days after sensitization does not produce evidence of sensitization of any type The guinea pigs have however undergone some internal immunologic transformation although the change has not proceeded either qualitatively or quantitatively to the point where the entire animal can overtly indicate a degree of delayed type hypersensitivity When lymph nodes from a sufficient number of animals are excised during the second or third day after sensitization and the washed cells are injected into recipients hypersensitivity of the delayed type can be detected However a ratio as high as 20 donors per recipient has to be used The donors during this time interval do not show any overt indications of hypersensitivity to intra

dermal injection of the specific antigen Also when the lymph nodes are excised teased apart and ground thoroughly so that the cells are disrupted circulating type antitoxin cannot be detected in the resulting suspension by rabbit intracutaneous tests

Originally the belief was held that delayed hypersensitivity could be produced primarily by intact microorganisms preferably alive and that soluble proteins induced Arthus reactions In the experiments on which this hypothesis was based relatively large doses of antigen were injected These observations do not contradict the present belief that delayed sensitivity is a step in antibody formation When a particulate antigen is used the soluble protein is apparently released in sufficiently small amounts to prolong and accentuate the period of delayed hypersensitivity and make this phase readily observable When a soluble protein is used for sensitization the amount injected can be so large that it tends to produce an early and prolonged development of Arthus reactions

An antibody from either the delayed or Arthus stage in the sensitization process therefore should be able to transfer passively hypersensitivity of its own type Cells from the lymph nodes during the period of delayed hypersensitivity transfer delayed hypersensitivity Cells from the lymph nodes at a later stage ultimately induce Arthus type hypersensitivity in a recipient Serum however can induce only Arthus type hypersensitivity in guinea pigs since the specific antibody associated with delayed hypersensitivity seems limited to the cells

It is possible that the processes of delayed and Arthus type hypersensitivity are not actually related or dependent upon one another but merely parallel one another in their development Thus with a sensitization dosage of 1 Lf toxoid 4 days are required to induce hypersensitivity of the delayed type and about 11 days to induce hypersensitivity of the Arthus type That delayed sensitivity is a stage in antibody formation is supported by the fact that induction of delayed hypersensitivity by means of the specific precipitate technique hastens the development of antibody and the appearance of an Arthus type reaction Thus delayed hypersensitivity may act as a booster mechanism in the production of antibody

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27

*Delayed Hypersensitivity and the Behavior of the Cellular Transfer System in Animal and Man**

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The demonstration of the cellular transfer of delayed hypersensitivity in the guinea pig by Landsteiner and Chase [9] and by Chase [1,3] presented the immunologist with a new approach to the study of the mechanisms underlying the delayed type of allergic inflammatory response In animal species the findings in relation to delayed sensitivity were soon confirmed [5,19,30] and the technique of cellular transfer was extended by Chase [5] and others [1,34,35] to the study of mechanisms underlying the synthesis of conventional serum antibody as well

Our interest in cellular transfer began with the relatively simple question directed to whether the transfer of delayed sensitivity could be accomplished in man The answer has been positive and direct in this laboratory [21,2] and subsequently in other laboratories employing a variety of bacterial [9,15,3,39] simple chemical [8,13] and viral [4] test materials associated with hypersensitivity of the delayed type The technique of transfer in man has employed leukocytes isolated and concentrated from the peripheral blood of sensitive donors and usually injected into or beneath the skin of the recipient's shoulder The challenging skin test with specific antigen is performed usually the following day on the volar surface of the forearm The use of viable leukocytes to effect transfer of delayed sensitivity in man had been predicted on the finding in animals by Chase [1,3] and others [37,41] that impairment of leukocyte viability abolishes their capacity to transfer sensitivity

The results obtained in man using viable leukocytes in general parallel those secured in animals Specifically the degree of sensitivity achieved in the recipient is conditioned by the degree of sensitivity of the donor

* This work from the Department of Medicine New York University College of Medicine and the Third (NYU) Medical Division of Bellevue Hospital New York, was conducted under the sponsorship of the Commission on Streptococcal Diseases Armed Forces Epidemiological Board and was supported in part by the Office of the Surgeon General Department of the Army and in part by a grant from the National Institute of Allergy and Infectious Diseases US Public Health Service (E 1254 C1)

and the volume of leukocytes transferred It is possible to fail to transfer sensitivity with inadequate volumes of leukocytes from adequately hypersensitive donors or with adequate volumes of leukocytes from only moderately sensitive donors Leukocytes obtained from nonsensitive donors or serum or erythrocytes obtained from sensitive donors are incapable of transferring delayed sensitivity [21,23]

In the human subject the delayed hypersensitive state conferred by leukocytes or leukocyte extracts has many similarities to that which occurs following natural infection it is immunologically specific it is generalized and involves the entire skin of the recipient wherever tested it is frequently as marked in intensity and has occasionaly been accompanied by a systemic response The local contribution of the leukocytes may be discovered by injecting antigen into an intradermal site of leukocyte deposition The general contribution of the recipient may be seen when quiescent leukocyte depots in the shoulder flare up at the time of a maximal reaction to antigenic challenge in the forearm [21,4,5]

In the course of these investigations it soon became apparent, however that the results observed in man differed from those secured in animals in several significant respects This chapter will consider the most apparent of these differences in general terms with a selective but not exhaustive survey of the literature In purpose this consideration may serve to suggest clues to underlying mechanisms which perhaps mediate at a different level the transfer of sensitivity in each species A more detailed account of the general problem of cellular transfer in man and its meaning in relation to altered tissue reactivity of the delayed type is available in several recent reviews

Differences relative to the number of donors required to confer sensitivity on the recipient and the volume of leukocytes necessary to accomplish this are admittedly difficult to quantitate However in the guinea pig 5 to 7 donors yielding a total of 0 7 ml of leukocytes may be required to sensitize one recipient [1,3] In the human subject one donor may be used to sensitize 4 or more recipients with from 0 1 ml to 0 5 ml of leukocytes per recipient [1,4,9] Indeed if antigen is injected into an intradermal site of leukocyte deposition then as little as 0 01 ml to 0 0 ml *of leukocytes may cause the appearance of at first local and then general*ized sensitivity in man [1,4,23] This type of dose response assumes some suggestive significance when the mass and the surface area available for dilution of the cells transferred into a 70 Kg adult human are compared with the mass and surface area of a 500–900 Gm guinea pig

The duration of the delayed hypersensitive state initiated following cellular transfer in the two species points up another line of division in relation to the effects observed The sensitivity conferred on the guinea pig by means of cells is a transient one varying from 3 to 5 days in dura

tion[1, 31] In contrast the delayed sensitivity transferred in man is more often of longer (months) than shorter (weeks) duration In many human recipients sensitivity may persist for anywhere from 6 months to 1 to 2 years following cell transfer[24] The prolonged duration of delayed sensitivity in the human recipient following cellular transfer has been confirmed recently by Good and his associates[11, 13, 18] and by Epstein and Kligman[8] More recent studies by Chase[4] however suggest that this species difference may not be as marked as it once appeared He finds that sensitivity transferred to the guinea pig also may commonly persist at high levels for months when large inocula of cells from exquisitely sensitive donors are used

The necessity for viable cells to insure transfer of delayed sensitivity in the guinea pig and in the rabbit is perhaps the most significant parameter in which the behavior of the cellular transfer system differs in these species and man This requirement would appear to be well established from the initial and subsequent findings of Chase[1, 5] and similar observations made in other laboratories[7, 37, 41] Possible exceptions to this experience in animals are suggested by the reports of Jeter and his associates[17, 8] and by Cummings Patnode and Hudgins[6] on the transfer of delayed sensitivity in the guinea pig with extracts of leukocytes disrupted by sonic vibrations

The ease and regularity with which extracts of leukocytes effect transfer in the human recipient would also appear to be well established The use of leukocyte extracts in the human recipient is effective in the transfer of delayed hypersensitivity to tuberculin to streptococcal proteins[24] and to diphtheria toxoid[8] The pattern of the results achieved is indistinguishable from that secured in man using intact viable leukocytes[23, 27] Freedman Fisher and Cooke[9] have recently confirmed these findings in man using sediments or extracts of disrupted leukocytes prepared by freezing and thawing or mechanical disruption to transfer tuberculin sensitivity

Moreover deliberate treatment of leukocyte extracts with the enzymes desoxyribonuclease or ribonuclease[4] or desoxyribonuclease plus crystalline trypsin[7] has not affected the biological activity of the factor or factors in human leukocytes concerned in the transfer of delayed sensitivity More recently it has been shown that transfer factor may be released in effective amounts from sensitive human leukocytes into cell free supernatant solutions under the relatively mild conditions of incubation at 37° C in serum for short periods[8]

Perhaps related to the efficacy of leukocyte extracts in the transfer of delayed sensitivity in man is the observation that human leukocytes may be stored in the frozen state for at least 5 months before extraction and leukocyte extracts may be stored in the frozen state for at least several weeks without effect on the capacity of such extracts to transfer sensi

tivity.[28,34] This and other data on transfer of sensitivity with human leukocytes held at 5° C and 37° C for 4 to 6 hours suggest a certain durability and hardiness of the biologically active material.[27]

Finally, differences in the experimental techniques employed in the transfer of delayed sensitivity have arisen as a secondary consequence of differences in species. For example, in the animal the leukocyte donors are of necessity actively sensitized by a variety of experimental means at varying intervals before securing the cells for transfer.[3,5,19,31] The human leukocyte donors have acquired their sensitivity as a result of natural infection from which they have recovered sometime in the past. It is of considerable importance to point out, however, that they do not suffer from the active disease related to the specific hypersensitivity under study.

In relation to the recipient, one can be reasonably certain that the experimental animal has never been exposed to the bacterium or test material under study before transfer of sensitivity. In the human subject, despite negative skin tests and adequate clinical and laboratory data, the possibility of previous exposure to antigen exists. While this is unlikely to be true of all recipients or of all antigenic systems studied, it is probable in a few instances that cellular transfer in man may be recalling an old sensitivity rather than conferring an entirely new one.[27]

The sources of leukocytes and the types of cells utilized for transfer also differ in the experimental animal and man. Most transfers in animals have employed leukocytes obtained from peritoneal exudates or from lymph nodes.[3,5,19,3] Nearly all of the transfers in man have employed leukocytes obtained from the peripheral blood.[10,15,21,24,8,39,40] This has given rise to the interesting finding in animals that lymph node cells are capable of transferring delayed sensitivity or serum antibody formation depending upon the mode of sensitization of the donor.[3] In the human, leukocytes obtained from the peripheral blood do not transmit the capacity for serum antibody formation while transferring marked delayed hypersensitivity to the same antigens. This is the case whether viable leukocytes[12] or leukocyte extracts[8] are employed. However, lymph node cells given to agammaglobulinemic recipients have been shown by Good and Zak[14] to transfer the capacity for serum antibody formation. Moreover, Martin, Waite and McCullough[29] have shown that in addition to transferring the capacity for serum antibody production, lymph node cells also transferred tuberculin sensitivity in the agammaglobulinemic patient studied by them.

The differences in the behavior of the cellular transfer system in man and in the experimental animal just cited may offer clues to the mechanism of transfer operative in each species. The necessity for intact viable leukocytes and the prompt appearance and short lived duration of transferred sensitivity in animals suggest that one is here dealing with a truly passive

transfer. The effects observed appear related to the presence of the transferred cells performing in the recipient a specific function ordinarily performed in the donor. This view would regard the recipient animal as a passive receptacle — an *in vivo* indicator of the functional contribution of the transferred cells. When such cells die, perhaps via a homograft rejection type of mechanism as suggested by the recent observations of Harris and Harris (see Chapter 37 of this volume), the capacity of the recipient to respond as the donor is at an end.

In the human recipient of sensitive leukocyte extracts we may be dealing with effects which are operative via two distinct and perhaps unrelated mechanisms. The prompt appearance of sensitivity within hours after transfer may be viewed to occur largely through the contribution of a readily available completely reactive antibody-like material. This type of response is in keeping with a passive transfer of biological activity in the conventional sense. However, the prolonged duration of sensitivity initiated by the transfer of leukocytes or their extracts requires a consideration of the contribution made by the recipient of the process.[24–27]

One of the more interesting and perplexing facets of the behavior of the subcellular transfer system in man relates to the widespread and prolonged effects initiated in the recipient following the introduction of a relatively small inoculum of leukocyte extract. This apparently simple maneuver activates in the recipient a mechanism which causes him to respond to the specific test material thereafter in the manner of the donor for periods beyond that which the leukocyte extract could be expected to survive. This viewpoint suggested analogies to other biological systems where self-replication or transformation appears superficially to achieve similar results. In consequence an experimental approach was undertaken which attempted to abolish the capacity of leukocyte extracts to transfer sensitivity by prior treatment with the enzymes desoxyribonuclease or ribonuclease.[2] Such treatment had no effect upon the capacity of leukocyte extracts to transfer sensitivity.

Additional similarities in the behavior of the cellular transfer system in guinea pig and man are becoming evident. I have referred earlier to Chase's[4] observations on the prolonged duration of transferred sensitivity (months) in the guinea pig. He also has observed two phases of sensitivity in the guinea pig following transfer, an early and a late phase.[4] The late phase of sensitivity differs from the initial events following transfer and Chase suggests it has a resemblance to a process akin to active sensitization. It should be noted that these similarities between the guinea pig and man are brought out when large volumes of cells obtained from exquisitely hypersensitive donors are transferred.

Evidence recently secured in human subjects[5] suggests that antibody of the conventional type is not transferred. Diphtheria antitoxin is not

detectable in leukocyte extracts effective in the transfer of specific sensitivity nor in the sera of recipients at the time of maximal delayed sensitivity to toxin or toxoid Moreover the addition of specific antigens (tuberculin diphtheria toxoid) to sensitive leukocytes before transfer does not neutralize the capacity to transfer delayed sensitivity

I do not believe the effects observed in man result from the transport of native antigen by the transferred leukocyte extracts This opinion is fostered by the transient life span of peripheral blood leukocytes the use of healthy leukocyte donors without the disease related to the specific sensitivity the inability to detect the formation of conventional serum antibody following transfer with peripheral blood leukocytes[12, 28] and the inability to transfer delayed sensitivity from man to animal with leukocytes or leukocyte extracts[7]

On the basis of information currently available[27] I would favor the interpretation that the function transferred is an expression of the imprint which the catabolism of the specific bacterium imposed upon the biosynthetic properties of the cells of the sensitive donor sometime in the past Peripheral blood leukocytes are incapable of further cell division and are short lived cells yet the sensitive donor s leukocytes are capable of transmitting sensitivity for many years after active sensitization has occurred This would suggest that blood forming stem cells and reticuloendothelial cells — altered in regard to the specific bacterium — continually confer on newly formed leukocytes before release to the peripheral blood a specific expression of this alteration which becomes manifest upon transfer to the nonsensitive recipient

If these assumptions are correct the material transferred may not be antigen in the usual sense but represent an intermediary product of the interaction of antigen and a specific cell population of the sensitive donor Such a postulated material may mediate the altered response of the sensitive donor thereafter to specific antigen It may in turn following transfer activate in the recipient similar stem cells or reticuloendothelial cells which are ordinarily concerned with attempts at the disposal of foreign particles such as bacteria

Whether the effects observed in the human recipient arise from the transmission of a heightened capacity for active sensitization in the presence of the transfer mechanism or whether conventional notions of active and passive sensitization will require modification cannot be decided at this time Also whether transfer factor is a peculiar type of antibody passed from cell to cell as a fully reactive entity[32, 33] or whether it has some more intimate role in altering the metabolism of the cells of the recipient in a manner similar to that in which the bacterium altered the metabolism of the cells of the donor remains for the present a pressing and intriguing question[24, 27]

In any event it is evident that the human subject is a more sensitive indicator of the biological activity of the factor or factors in leukocyte extracts concerned with the transfer of delayed hypersensitivity. This allows for attempts at the identification and perhaps quantitation of transfer factor. A step in this direction has been taken in the enzymatic [4] and in the immunologic [5] experimental approaches to this problem.

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GENERAL DISCUSSION

JUSTINE S GARVEY (Pasadena California) Dr Pappenheimer s remarks about theories of antibody formation prompt my remarks about antigen persistence particularly as it is related to one theory — the template theory Dr Dan Campbell and I have found that antigen persists for a very long time in the rabbit liver We have been able to detect it very easily for over a year s time, and by extrapolating our results there would still remain several hundred molecules per liver cell even at 3 years[1,2] More than just studying the persistence of a label, we have identified the label, the S_{35} sulfanilic acid group with the native protein[3] and also have found that upon injection into guinea pigs this material is still antigenic[1] Even though the rabbit may be an improper animal for some experiments, one can extend these results as probably involving humans also Antigens are undoubtedly retained for a very long period of time in all species and these findings erase the old objection to the template theory

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ELVIN A KABAT (New York New York) I should like to ask Dr Lawrence what evidence he has that he has produced a delayed hypersensitivity to diphtheria toxoid itself and not to any of the numerous contaminating proteins present in it which very successfully induce anamnestic responses in humans

CHAIRMAN EISEN We have a comment and a question one directed to Dr Pappenheimer and one directed to Dr Lawrence Let me ask Dr Pappenheimer if he would like to reply to Dr Garvey s comment regarding the persistence of antigen in relation to the trapping mechanism he has described

DR PAPPENHEIMER The findings of Drs Garvey and Campbell on the prolonged persistence of antigen are interesting and important Dr Marion Koshland s findings may also be mentioned at this point Dr Koshland succeeded in demonstrating persistence of diphtheria toxoid in guinea

pig tissues as long as . months after its injection even in the presence of high titers of circulating antitoxin

I might also comment on Dr Kabat s question to Dr Lawrence Lawrence and I demonstrated the specificity of induced hypersensitivity to toxoid in man by showing that delayed skin reactions were strikingly reduced in size after specific removal of the toxoid component by precipitation with an antitoxin which had previously been shown to contain no more than traces of other antibodies

DR KABAT I should like to say that we have never seen a sample of antitoxin including Dr Pappenheimer s sample which contained so called nonprecipitating antitoxin in which we could not find precipitating antibodies to other impurities

DR LAWRENCE The question directed at the use of purified diphtheria toxoid as antigen in the cellular transfer system has been answered by Dr Pappenheimer I should like to add that the tuberculin and streptococcal proteins used in earlier studies on cellular transfer in man were chosen as indicators of the appearance of delayed hypersensitivity The biological event was the deliberate focus of interest rather than the purity of the test materials used to signal its occurrence This choice was appropriate for the stage of development the problem had reached at that time Implicit in this approach however has been the prospect that the delayed hypersensitive state may be amenable to quantitation when the factor(s) in human leukocytes concerned in the transfer of sensitivity is identified

PAUL H MAURER (Pittsburgh Pennsylvania) I should like to comment on two sets of experiments one done in collaboration with Dr Mansmann at the University of Pittsburgh and the other done in collaboration with Dr Benacerraf of New York University

I have taken the opposite position initially that it should be possible to detect antibody before one skin tests the guinea pigs that have been injected with the specific precipitates As soon as one skin tests guinea pigs that have been injected according to the Uhr Salvin Pappenheimer technique a secondary response is initiated as Dr Pappenheimer has explained

We therefore employed the very elegant technique of Dr Ovary the passive cutaneous anaphylaxis reaction The experiment was set up so that the precipitates used for immunization were made with seven times recrystallized egg albumin which is a purified protein but the PCA reaction was performed with impure once recrystallized ovalbumin which has other proteins in it We could not detect any PCA reaction which is sensitive to 0 003 μg of antibody even up to 60 days after the initial injection

GENERAL DISCUSSION

JUSTINE S GARVEY (Pasadena California) Dr Pappenheimer s remarks about theories of antibody formation prompt my remarks about antigen persistence particularly as it is related to one theory – the template theory Dr Dan Campbell and I have found that antigen persists for a very long time in the rabbit liver We have been able to detect it very easily for over a year s time and by extrapolating our results there would still remain several hundred molecules per liver cell even at 3 years[1 2] More than just studying the persistence of a label we have identified the label, the S_{35} sulfanilic acid group with the native protein[3] and also have found that upon injection into guinea pigs this material is still antigenic[3] Even though the rabbit may be an improper animal for some experiments one can extend these results as probably involving humans also Antigens are undoubtedly retained for a very long period of time in all species and these findings erase the old objection to the template theory

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ELVIN A KABAT (New York New York) I should like to ask Dr Lawrence what evidence he has that he has produced a delayed hypersensitivity to diphtheria toxoid itself and not to any of the numerous contaminating proteins present in it which very successfully induce anamnestic responses in humans

CHAIRMAN EISEN We have a comment and a question one directed to Dr Pappenheimer and one directed to Dr Lawrence Let me ask Dr Pappenheimer if he would like to reply to Dr Garvey s comment regarding the persistence of antigen in relation to the trapping mechanism he has described

DR PAPPENHEIMER The findings of Drs Garvey and Campbell on the prolonged persistence of antigen are interesting and important Dr Marion Koshland s findings may also be mentioned at this point Dr Koshland succeeded in demonstrating persistence of diphtheria toxoid in guinea

pig tissues as long as [illegible] months after its injection even in the presence of high titers of circulating antitoxin

I might also comment on Dr Kabat's question to Dr Lawrence Lawrence and I demonstrated the specificity of induced hypersensitivity to toxoid in man by showing that delayed skin reactions were strikingly reduced in size after specific removal of the toxoid component by precipitation with an antitoxin which had previously been shown to contain no more than traces of other antibodies

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The results are shown in the following table

TABLE I

Serum Obtained Before Testing	Skin Reactions (Average) in Millimeters Immediate	Delayed	Tests for Antibody Agar diff (Preer)	PCA	Coprecipitation
D10	8 x 8	35 x 35	—	—	—
D20	8 x 8	19 x 19	—	—	—
D30	4 x 4	15 x 15	—	—	—
D60	7 x 7	11 x 11	—	—	—

With Dr Benacerraf we employed two types of cross reacting systems one involving two native proteins such as human serum albumin and bovine serum albumin When guinea pigs immunized against human serum albumin were tested with bovine serum albumin they showed a delayed reaction to the BSA but no immediate reaction Another system involved cross reacting antigens which have been artificially prepared, namely acetylated human serum albumin and human serum albumin. When guinea pigs were immunized with N acetylated HSA only a delayed reaction was observed when skin testing with HSA Dr Benacerraf is not here so I shall say nothing about the interpretations of these results which appear quite confusing I know Dr Pappenheimer has discussed them with Dr *Benacerraf*

MERRILL CHASE (New York, New York) I am sure that Dr Dienes would be pleased were he here today for his work and its interpretation which impressed so few people at the time has now shown itself justified namely that when a protein is injected into the skin the first response of an animal is the appearance of a delayed type reaction It is now confirmed amply Most important are experimental manipulations that serve to prolong the time during which delayed type responses are observed to persist before the onset of [illegible] in the circulation and the consequent change in the characte[illegible] reaction The new procedures consist in a use of much smal[illegible] es of protein in making the initial antigenic stimulation and al[illegible] masking of areas of the antigenic protein by [illegible] them w[illegible] antibody

I believe [illegible] that [illegible] made [illegible]ienes was f[illegible]ulty [illegible] may [illegible] into p[illegible] Is it true as he [illegible] converted into [illegible] Intra dermal [illegible] ding this [illegible] ius type [illegible] er a[illegible] ficit

delayed type reactions In contrast to proteins however with application of certain allergenic chemicals that are soluble in oils and can be used for percutaneous application it can be shown that the delayed type of hypersensitivity persists undiminished even after antibody appears and vitiates the use of allergen protein conjugates injected intradermally to elicit delayed type reactions In this instance coexistence of two types of sensitivity is easily demonstrable Consequently there appears to be no reason to make the assumption that one type of hypersensitivity becomes converted into the other type

Whether the presence of two forms of hypersensitivity represents a clonal variation in cell types some being capable of going onward to develop antibody and others persisting in their erstwhile function we cannot of course say but in any event one can ascertain that the degree of contact type hypersensitivity does not fall as antibody production commences

Adolph Rostenberg Jr (Chicago Illinois) I should like to disagree mildly with some of the implications in the paper by Dr Kaplan and in the discussion by Dr Salvin I have no doubt as to the correctness of their findings nor do I have any doubt that this is a different type of reaction than the Arthus phenomenon I just wonder what the correct interpretation is of the word delayed

That the reaction is delayed temporarily of course is obvious but the implication that this means it is the same as tuberculin type hypersensitivity seems to me at least at present unwarranted I might point out Klemperer s wise caution that pathologic identity is no evidence for pathogenetic relatedness Everyone here is aware of the difficulty of developing tuberculin type hypersensitivity with tuberculoprotein The various efforts in that direction I do not have to enumerate

Every allergist here has injected minute amounts of protein solutions in various types of skin testing and I would doubt if he has seen tuberculin or delayed type hypersensitivity develop even temporarily as a consequence of such intracutaneous introduction From the practical side if Dr Kaplan s and Dr Salvin s findings were true it would be a serious difficulty with the epidemiologic value of delayed type skin testing One of the virtues of delayed type skin testing is that you do not develop the delayed type by the introduction of this material

So I would rather take the Scotch verdict of Case not proved and hold this to be as yet a different variety of sensitivity or one which should be unlabeled or at least labeled differently from the classic tuberculin type of hypersensitivity I will take refuge in a quotation from Santayana who said Skepticism is the chastity of the intellect and should not be surrendered lightly to the first comer

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*Delayed Hypersensitivity in Agamma-globulinemia**

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Since Bruton s description in 195 [1] of a child suffering from congenital agamma globulinemia this disease has been looked upon in our laboratory as an experiment of nature which we feel provides a unique opportunity for the investigation of various aspects of immunity and hypersensitivity The agamma globulinemic state is clinically characterized as one in which *striking susceptibility to bacterial infection is associated with an almost* complete absence of gamma globulin from the serum In addition antibodies are absent from the blood and tissues and failure of antibody formation in response to even the most intense antigenic stimulation is characteristic Two major forms of persistent agamma globulinemia have been described In children the disease most commonly appears to be hereditary being transmitted as a sex linked recessive trait This form occurs only in male children In addition it occurs in adults as a spontaneously acquired disease without known etiology [2, 8, 10, 12, 20, 22]

In other clinical diseases gamma globulin concentration in the serum may be low but in most of these instances excessive losses or more rapid catabolism of gamma globulin accounts for a low concentration of this protein in the serum In each of the patients to which we shall refer today it has been established by immunochemical means that the gamma-globulin concentration in the blood is extremely low In the children it ranged from 1 5 to 14 mg per cent and in the adults between 11 and 40 mg per cent Furthermore determinations of gamma globulin half life in the serum following the intramuscular injection of gamma globulin concentrates have revealed a decay rate ($T\frac{1}{2}$ = 27 to 35 days) for the gamma globulin in these patients somewhat in excess of the estimates for normal persons In addition extensive immunological studies on each of these patients have revealed complete failure or a marked deficiency in

This study from the Pediatric Research Laboratories of the Variety Club Heart Hospital University of Minnesota has been aided by grants from the U.S Public Health Service Minn sota Heart Association American Heart Association Minnesota Division of the Arthritis and Rheumatism Foundation and Minnesota Division of the American Cancer Society

capacity to form circulating antibody regardless of the type of antigen given, the route of administration employed, the time relationship to prior antigenic injections or method used to demonstrate circulating antibody It was further discovered independently in both our laboratories and those of the Harvard group that these patients lack plasma cells and fail to form plasma cells in their bone marrow and lymphoid tissue in response to the most intensive antigenic stimulation [2, 5, 6] During the past five years we have had an opportunity to study 22 cases of agamma globulinemia fitting this clinical and laboratory definition It is from these cases that our material to be presented today has been drawn

From our early investigations we concluded that these children were deficient not only in capacity to form circulating antibody but also in capacity to develop delayed allergy of the tuberculin type This conclusion was based on studies comparing the incidence of delayed type allergy against tuberculin streptococcal products and pneumococcal products of patients with agamma globulinemia to the reactivity of normal persons and other hospitalized patients In these studies a striking difference in the two groups was apparent. For example none of the 2 agamma globulinemic patients studied showed sensitivity to streptokinase streptodornase none showed delayed allergy to pneumococcal vaccine tuberculin and histoplasmin whereas among the normal children normal adults and patients hospitalized for study or treatment of other diseases, a high percentage showed delayed allergy to both streptococcal and pneumococcal antigens

Reactivity to tuberculin and histoplasmin is relatively uncommon in our area so that the negative tuberculin and histoplasmin reactions are not particularly meaningful To the contrary normal children and adults in our area have a uniformly high incidence of sensitivity to both streptococcal and pneumococcal products Since the agamma globulinemic patients have had a similar environmental exposure and indeed were frequently shown to be infected with these organisms it appeared to us that the defect responsible for the disease must include the pathway for the development of delayed hypersensitivity

Since it appeared possible that cellular events occurring in the course of the delayed allergic reaction might be defective we carried out a series of experiments to test this hypothesis These studies showed that it was regularly possible to induce sensitivity to streptokinase streptodornase by the subcutaneous injection of leukocytes from highly sensitive normal persons into the agamma globulinemic patients The transfer of hypersensitivity was successfully accomplished in three children with congenital agamma globulinemia and in a male adult with the acquired form of the disease Two other agamma globulinemic patients were followed as controls with repeated skin testing using streptococcal and pneumococcal

antigens during the year subsequent to the original transfer Neither of these patients spontaneously developed sensitivity, and during this same period two of the three patients that had been passively sensitized reverted to a negative reaction However in one of the children the transferred sensitivity persisted for more than years In the two agamma globulinemic patients who lost their transferred sensitivity reversion occurred between 4 months and 6 months following transfer in one and between months and 4 months following transfer in the other We interpreted this observation as evidence that these patients with agamma globulinemia are able to express and sustain delayed allergy

During the course of our investigations several observations led us to question the apparent inability of these patients to *develop* delayed hyper sensitivity For example we observed that these patients respond in a normal manner to the injection of Gram negative bacterial endotoxins Thus in each of the agamma globulinemic patients whom we studied the usual induration erythema and local tenderness developed following intradermal injections of *Escherichia coli*, meningococcus, typhoid or Pseudomonas endotoxins into the skin In all instances this local reaction was every bit as vigorous as that observed among control patients In addition the agamma globulinemic patients tested showed capacity to develop chills, fever headache malaise characteristic leukocyte changes and acute phase reactions following the intravenous injections of Gram negative bacterial endotoxins These systemic adjustments were no different among these patients than in other patients and normal individuals having normal immunologic mechanisms This reaction has been considered by some [5] to be a form of delayed allergy As an infant, one of our children with congenital agamma globulinemia developed a severe monilial dermatitis over the diaper area The intensity of the associated inflammatory reaction suggested that this child had indeed developed delayed allergy to *Candida albicans* This reaction healed completely when the Candida infection was controlled by treatment with Mycostatin(R) In addition this patient showed a strongly positive reaction to skin testing with Candida antigen Another patient in our group who originally was reported to have an erythematous reaction to tuberculin without induration has subsequently developed tuberculin hypersensitivity of classical type

Zinneman in 1953 [9] *was the first to describe an adult suffering from* acquired agamma globulinemia who was tuberculin positive Since then another adult with the acquired form of agamma globulinemia having calcified pulmonary lesions was shown to have a positive histoplasmin reaction [24] Sanford *et al* [23] also reported in patients with acquired agamma globulinemia the capacity to react to certain bacterial antigens Kulneff Pedersen and Waldenstrom [17] reported that children with

congenital agamma globulinemia may be uneventfully vaccinated with BCG and that they develop a positive tuberculin reaction following such vaccination This finding was subsequently confirmed by Porter[1] who in addition transferred the delayed reaction to immunologically normal persons Because of the confusion inherent in these observations we have systematically studied this question in a group of children and adults suffering from the congenital and acquired forms of agamma globulinemia In these studies we have been able to actively sensitize agamma globulinemic patients to 2 4 dinitrofluorobenzene (DNFB) to diphtheria toxoid to horse gamma globulin and to Ascaris polysaccharide antigen Sensitization to 2 4 DNFB was accomplished by administering as a patch test a vesicant dose (a patch soaked in a 1 10 dilution of 2 4 DNFB in olive oil and acetone) Testing of sensitivity is then achieved by applying a patch test of 2 4 DNFB in less than vesicant concentration (1 1000 to 1 10 000 dilution) in the same vehicle The application of the vesicant dose regularly results in the development of delayed hypersensitivity in immunologically normal individuals Sensitization is not produced by the application on several occasions of the test dose of 2 4 DNFB Using large numbers of viable white cells isolated from the peripheral blood we were able to transfer this reaction by injection of leukocytes from either sensitized agamma globulinemic or sensitized normal patients to unsensitized normal individuals in four out of five attempts The inability to sensitize with the test dose employed even on repeated application supports our conclusion that we have achieved passive transfer of this form of delayed allergy

On three different occasions we have attempted to transfer this reaction using large volumes of plasma from highly sensitized agamma globulinemic patients On the first attempt 150 ml of plasma was used on the second and third 200 ml was employed The plasma injections were given intravenously to immunologically normal recipients Though the donors in all three instances had severe delayed inflammatory reactions to the skin test dose of 2 4 DNFB their serum injected into the normal recipient failed to transfer the delayed allergic reaction Similarly the agamma globulinemic state does not preclude the development of delayed allergy to diphtheria toxoid Using specific precipitates of diphtheria toxoid prepared in the zone of antibody excess by A M Pappenheimer Jr and administering these intradermally into the skin of the shoulder in amounts sufficient to provide a total dose of 5 Lf of toxoid we have observed the active development of delayed allergy in patients with agamma globulinemia in every instance Sensitization was achieved in most instances by one series of injections of the specific precipitates These were administered as a saline suspension of the precipitate The antibody was a horse gamma globulin concentrate containing a high titer of anti

toxin The suspension of precipitates was injected at five sites and skin testing performed weeks later with 0 1 cc (0 009 Lf) of diphtheria toxoid (Schick control Massachusetts State Board of Health) The experimental approach was patterned after the studies in guinea pigs recently reported by Uhr *et al* [28] In agamma globulinemic patients this procedure resulted in the development of delayed hypersensitivity while simultaneous serological study revealed no antitoxin formation In normal children immunization with these materials resulted in the development of delayed hypersensitivity in approximately 60 per cent of cases irrespective of the level of pre existing antitoxin Some normal children however responded to the injection of specific precipitates by production of significant amounts of circulating antitoxin Surprisingly and very strikingly these children did not develop delayed allergy to the toxoid Repeated stimulation with specific precipitates resulted in development of delayed allergy in some children who did not respond to the first immunization and produced in most of the normal children and agamma globulinemic patients a significant increase in the degree of sensitivity in those responding initially This form of delayed allergy was also transferred to non sensitive recipients by the subcutaneous injection of viable leukocytes from sensitized agamma globulinemic as well as from actively sensitized normal donors In these experiments we observed that passive transfer of either form of sensitivity from both immunologically normal sensitized persons and sensitized agamma globulinemic patients required large numbers of viable peripheral blood cells Although we have made several attempts we have not yet been able to transfer these forms of delayed allergy with frozen thawed cell suspensions according to the method of Lawrence [18, 19] We consider our failure to be a function of inadequate methodology

Strikingly the development of sensitization to diphtheria toxin with the precipitates prepared in this way was not associated with sensitization to the horse globulin also contained in the precipitates Sensitization to horse gamma globulin however was achieved in both normal and agamma globulinemic recipients by the use of specific precipitates of horse gamma globulin with rabbit antisera prepared against horse gamma globulin The specific immunological precipitates in these studies were also prepared in the region of antibody excess and administered by a schedule similar to that used for the diphtheria toxoid antitoxin precipitates This delayed allergic reaction was also transferred to nonsensitive normal recipients by using viable leukocytes obtained from the peripheral blood

Subsequent to these observations we have found skin sensitivity to mumps skin test antigen to be present in four agamma globulinemic patients who had previously suffered clinical infection with mumps virus

It is striking in this regard that no demonstrable complement fixing antibody was present in any of these sera

With respect to other forms of allergy we have not found among the agamma globulinemic patients any completely convincing evidence of the existence of atopic allergy However two of the children have suffered from a dermatitis which clinically would be called atopic dermatitis The lesions developing are variable but regularly involve the cheeks antecubital and popliteal areas and are associated with significant itching and mild eosinophilia However, extensive efforts to locate an allergen which will produce a wheal and erythema reaction when injected into the skin have been negative Consequently it has been impossible to prove the occurrence of atopic allergy in our agamma globulinemic patients However an observation made by John Vaughan at the Medical College of Virginia[27] may be of greater significance Vaughan has studied a patient with acquired agamma globulinemia who had clinical manifestations of nonseasonal rhinitis This man had only .3 mg per cent of gamma-globulin and failed to respond immunologically to a variety of stimuli The patient had a positive wheal and erythema reaction to house dust, and passive transfer of the skin sensitivity to a nonsensitive recipient was achieved by the classical Prausnitz Kustner reaction These two observations have provoked us to attempt to achieve immediate wheal and erythema sensitization in our agamma globulinemic patients by injecting them with Ascaris antigens according to the method of Kailin.[15,16] Thus far although the technique works well in producing wheal and erythema sensitivity in immunologically normal volunteers the agamma globulinemic children studied have developed only delayed allergy after repeated injections of Ascaris antigens

As has been repeatedly emphasized agamma globulinemic patients deal with certain virus infections much more effectively than they do with the majority of bacterial infections due to the so-called extracellular bacterial pathogens For example they may develop measles and chickenpox express these diseases in the usual way with typical rash and recover from the infections at or near the usual time of recovery of immunologically normal persons Furthermore they have a striking capacity to resist recurrences of certain virus infections A good example is vaccinia infections where agamma globulinemic patients of both our group and the Harvard group[4] have been successfully vaccinated and have shown accelerated response to revaccination even though no circulating antibody was detectable in the serum.

It seems to us entirely possible that a certain primitive immunological capacity which is retained in the agamma globulinemic patient makes it possible for him both to develop delayed allergy and to resist certain virus infections Further study of this point is needed

Finally, among the agamma globulinemic patients we have studied in both the congenital and acquired groups we have found diseases ordinarily classified among the collagen group or the so called diffuse fibrinoid vascular diseases to occur with inordinate frequency [7] For example six of our patients have a syndrome of rheumatoid arthritis which in some cases cannot be clinically separated from rheumatoid arthritis as it occurs among the immunologically capable population in our community In some instances, the disease has been somewhat milder than that we have observed among the immunologically active persons but this is by no means a uniform finding In addition we have observed an apparently agamma globulinemic child [7] whose rheumatoid disease was clinically similar to Still s disease and who ultimately died with lesions characteristic of certain diffuse fibrinoid vascual diseases Indeed the pathologist made a diagnosis of disseminated lupus erythematous on the basis of his histological study Arild Hanson [22] had a child with apparently congenital agamma globulinemia who had perfectly typical dermatomyositis by clinical and biopsy criteria and Von Gelder [23] studied a female child who suffered from a disease clinically reminiscent of scleroderma Janeway and Gitlin [13, 14] likewise have encountered this high frequency of joint and connective tissue disease among patients with congenital agamma globulinemia It seems to us that these observations are more than provoking in light of current theories concerning the nature and pathogenesis of the connective tissue diseases

Comments

These studies present convincing evidence that agamma globulinemic patients can regularly be made to develop delayed allergy even without antibody formation when stimulated properly However the apparent inability of these patients naturally to develop sensitivity to streptococcal and pneumococcal products appears to be in conflict with the observation that delayed sensitivity may be regularly and without apparent difficulty developed in these patients by artificial means These patients are also capable of developing delayed allergy during the natural course of various infections as has been shown by the natural development of tuberculin sensitivity and its development after BCG infection Sekzer s [4] patient illustrates the ability to develop sensitivity during the course of histoplasmosis infection Delayed skin allergy to mumps antigen also occurs in some agamma globulinemic patients following infection with mumps virus Agamma globulinemic patients develop immunity to reinfection following an initial episode of certain virus diseases like measles chicken pox and vaccinia Could it be that this form of immunity then is a function of a primitive immune response capable of resulting in delayed

allergy which is retained by these patients to a remarkable degree. Certainly this circumstantial evidence suggests that this hypothesis is worthy of investigation.

If agamma globulinemic patients then retain the immunological capacity which enables them to develop delayed allergy and retain capacity to resist virus infection, why do they not develop delayed allergy to streptococcal and pneumococcal products following infections with these microorganisms? Study of these infections in our agamma globulinemic patients suggests a possible explanation for this paradox. On several occasions we have attempted to watch an agamma globulinemic patient without antibiotic treatment during a mild streptococcal or pneumococcal respiratory infection. This has uniformly led to progression of the disease to involve the lungs, blood stream, deep tissues, or meninges — infections which demand complete elimination of the organisms from the tissues by intensive antibiotic therapy. It is possible that the reason delayed allergy to streptococcal and pneumococcal products was not observed in agamma globulinemic patients is that with their lack of capacity for serological resistance the survival of the agamma globulinemic patient infected with one of the extracellular pathogens requires treatment with antibiotics resulting in elimination of these organisms from the tissues — an event precluding development of delayed allergy to the products of these organisms. That various fibrinoid diseases occur with inordinate frequency in patients with agamma globulinemia seems well established. The observation that capacity to develop delayed allergy is retained by these patients as clarified in this report, together with the suggestion by our data that in normal persons delayed allergy and development of classical immune response are somewhat mutually exclusive, suggests the possibility that it is delayed allergy and not classical immunity which is responsible for the occurrence of rheumatoid arthritis and other fibrinoid diseases in the agamma globulinemic patients. We interpret this observation as evidence that certain forms of rheumatoid disease and fibrinoid diseases are not dependent on production of excessive amounts of circulating antibody but rather probably depend on other mechanisms intact in the agamma globulinemic patient. It is possible that these mechanisms involve delayed allergy at some stage.

The inability of two of our patients to reject homotransplants of skin while retaining capacity to develop delayed allergy to 2,4 DNFB, diphtheria toxoid and horse gamma globulin certainly questions the hypothesis that it is delayed allergy alone, or in its usual form, which is responsible for the homograft reaction.

Summary

It may be concluded from our studies that although individuals with agamma globulinemia suffer a profound disturbance in the immune response, they retain capacity to develop delayed allergy. This observation would tend to dissociate the mechanism of development of delayed allergy from that involved in classical immune response resulting in formation of circulating antibody and gamma globulin.

Speculations concerning the role of delayed allergy in resistance to certain virus infections and to the development of fibrinoid necrosis and the homograft reaction are presented.

The observations presented here would be consonant with the theory presented in this monograph by Pappenheimer that delayed allergy is a stage in the development of classical immunity and antibody production. If such is the case, the agamma globulinemic patient is able to initiate but not complete the immune response.

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*Metabolic Activities of Isolated Lymph Node Cells**

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In recent years much of research and teaching concerned with delayed pe hypersensitivity has been influenced by the feeling that an event of ntral importance in this phenomenon is damage to the sensitized host s lls as a consequence of interaction between sensitizer and a structurally implementary substance in or on the surface of these cells The principal perimental support for this feeling has come from experiments in which imologous antigens inhibit migration and proliferation of explanted cells rived from hosts with delayed type hypersensitivity to a greater extent an they inhibit cells derived from nonsensitive hosts In the thirty years hich have elapsed since observations of this kind were first described[1, 21] ie original experiments have been confirmed by many investigators[14, 19, 20, 22] Many others however have been unable to reproduce these adings[2, 9] The difficulties encountered in placing these observations on firm basis may arise in part from the fact that the behavior of explanted ssue is difficult to quantitate and partly from the possibility that donor umal hypersensitivity may have to be extraordinarily intense in order or the altered response to be detected *in vitro* There remains in any vent considerable difficulty in reproducing these crucial observations id there is accordingly a great need for an isolated cell system which an be manipulated readily and reproducibly and whose functions can be ieasured with precision

Aside from the very important general issue involved an explanted ssue response suggests the possibility that a model system might be found or study of delayed type hypersensitivity responses independently of he intact animal The latter consideration assumes particular importance

* This work from the Division of Dermatology Department of Internal Medicine Vashington University School of Medicine and the Barnard Free Skin and Cancer Iospital St Louis has been supported in part by grants from the National Institute f Allergy and Infectious Diseases National Institutes of Health U.S Public Health ervice and in part by a contract (DA 49-007 MD-69) with the Research and Development Division Office of the Surgeon General Department of the Army ponsored by the Commission on Cutaneous Diseases Armed Forces Epidemiological Board

when one realizes that current efforts to study these allergic reactions are largely frustrated by the circular dilemma wherein there is both insufficient information available for development of analytical procedures and, at the same time a scarcity of suitable assay procedures which hinders the accumulation of information Just about the only relevant procedure now available for direct analysis of delayed type allergic skin responses is the skin reaction itself in the intact animal It is unnecessary to emphasize the intrinsic disadvantage of using the complete and complex phenomenon itself as the sole assay for analysis of the steps which determine it.

In view of the foregoing no further justification is required for our preoccupation with the metabolic behavior of cells isolated from donors with delayed type hypersensitivity The metabolic activities to be discussed reflect protein synthesis permeability to sugars respiration, glycolysis and phosphoprotein synthesis One or more of these functions would be expected to be altered in cells injured to the point where their behavior in tissue culture is grossly abnormal The delayed type hypersensitivity response we are interested in is contact skin sensitivity, specific *for the dinitrobenzene* * *group Hence our choice of lymph node cells* is an obvious one and stems from the basic observations of Chase concerning the capacity of such cells to transfer contact skin sensitivity from sensitized donors to normal recipients [5]

The studies we wish to describe are concerned with two questions

(1) Are metabolic functions of lymph node cells isolated from donors with DNP specific contact skin sensitivity altered to a different extent than are the same functions of corresponding cells of nonsensitized animals when the cells are exposed to DNP sensitizers *in vitro?*

( ) Can a population of isolated lymph node cells which are competent to transfer contact skin sensitivity be distinguished from populations of similar cells which are incompetent to execute such a transfer?

We may state at the outset that considerable information regarding some metabolic activities of these isolated cells has been obtained The results obtained thus far do not however permit us to distinguish between cells from DNP sensitive animals and cells from guinea pigs that are insensitive to this simple grouping

Cells from four different kinds of donors have been studied

(1) Guinea pigs sensitized with DNFB in complete Freund s adjuvant † These animals have a high degree of DNP specific contact skin sensitivity and their lymph node cells can be rendered competent for transfer of

* The following abbreviations are used DNP 2,4-dinitrobenzene or 2,4-dinitrophenyl group DNFB 2,4 dinitrofluorobenzene DNCB 2,4 dinitrochlorobenzene DNBSO$_3$ 2 4-dinitrobenzenesulfonate potassium salt DNP lysine N 4-dinitrophenyl lysine DNP protein protein substituted with 4 dinitrophenyl principally on NH groups of lysine residues

† *Mycobacterium butyricum* generously donated by Dr Melvin Cohn was used

sensitivity by appropriate specific stimulation These donors have concomitant anaphylactic sensitivity also DNP specific

(2) Guinea pigs sensitized with dinitrophenyl protein conjugates (DNP protein) in complete Freund s adjuvant These animals have a high degree of DNP specific anaphylactic sensitivity but do *not* have contact skin sensitivity

(3) Guinea pigs injected with Freund s adjuvant alone

(4) Uninjected normal guinea pigs

All injections of sensitizers and of control materials were into footpads Cell suspensions were obtained subsequently by teasing from axillary popliteal and inguinal nodes In the case of uninjected animals additional lymph nodes were used as well In a given suspension of cells determination of optical density at 660 mμ affords a rapid reproducible measure of number of cells and dry weight of cells per milliliter of suspension (Figure 1)

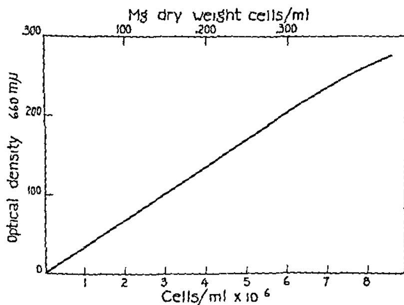


FIGURE 1 Relationship between optical density and number of lymph node cells and dry weight of cells per milliliter of suspension Medium modified Krebs-Ringer balanced salt solution

Amino Acid Incorporation into Cell Protein

C^{14} labeled amino acids are incorporated into the protein fraction of isolated lymph node cells at a linear rate for the first 1 to hours of incu

bation Justification for considering the C^{14} labeled product isolated to be protein rests on its having the following properties

(1) Insoluble in cold and hot (95° C) 5 per cent trichloroacetic acid

(2) Insoluble in warm (45–50° C) 3 1 ethanol ether

(3) A nitrogen content of 14 3 per cent*

(4) Treatment with trypsin and chymotrypsin, or with pepsin, leads to essentially complete solubilization of incorporated isotope in cold 5 per cent trichloroacetic acid

The maximal rate of incorporation of C^{14}-uniformly labeled L valine is reached at an initial extracellular concentration of 6×10^{-5} M L valine and amounts to 1 mμmole L valine/mg protein/hour For C^{14} methyl labeled L methionine the maximal rate of incorporation is attained at an initial extracellular concentration of 6×10^{-4} M L methionine and is 1 7 mμmole/mg protein/hour The rate of amino acid incorporation into protein is inhibited about 90 per cent by the addition of iodoacetate (10^{-3} M) or sodium azide (10^{-2} M) or by anaerobiosis Omission of glucose produces about 50 per cent inhibition in rate of incorporation

Previous work has shown that a number of simple dinitrobenzene sensitizers enter isolated lymph node cells readily, indeed some of the more potent sensitizers are concentrated by these cells[13] Since DNP sensitizers are moreover effective protein reagents[11] they may be expected to be potent inhibitors of many metabolic functions This is indeed observed in respect to amino acid incorporation into protein However cells from donors with a high degree of DNP specific contact skin sensitivity and cells from nonsensitive donors were inhibited to the same extent when incubated for 3 to 17 hours with several dinitrobenzene sensitizers over a wide range of concentrations A few representative results are shown in Table I In one experiment addition to the incubation mixtures of fresh autologous serum as a source of complement or other possible required factors did not significantly influence these results

For the simplest DNP sensitizers the order of toxicity is DNFB > DNCB ≫ $DNBSO_3$ This order correlates well with rates of reaction of these substances with protein amino groups and with SH groups[3] Consistent also with this order of toxicity is the fact that DNCB is concentrated by lymph node cells whereas $DNBSO_3$ enters these cells to a much lesser extent[13] DNP lysine does not react chemically with proteins and is not a sensitizer Possibly its slight toxicity may be due to its functioning as a lysine analogue

A preliminary attempt was made to determine whether lymph node cells of sensitive donors react differently than do the corresponding cells of nonsensitive donors following exposure *in vivo* to homologous sensitizer In these experiments guinea pigs were sensitized with DNFB in complete Freund s adjuvant Control guinea pigs were injected in foot

* We wish to thank Dr Fred Karush for the micro Kjeldahl analysis

TABLE I C^{14} LABELED AMINO ACID INCORPORATION INTO PROTEIN OF ISOLATED LYMPH NODE CELLS INCUBATED WITH 2,4-DINITROBENZENES*

2,4-Dinitrobenzene Added	Initial Concentration	Cells† from Sensitive Donors: Amino Acid Incorp	Cells† from Sensitive Donors: Difference	Cells† from Control Donors: Amino Acid Incorp	Cells† from Control Donors: Difference	Incubation Conditions‡
	M/L × 10^{-4}	cpm	%	cpm	%	
0	—	170		186		17 hr prior to addition of C^{14} methyl L-methionine
DNFB	9	32	−81	34	−82	
0	—	2400		2615		3 hr prior to addition of C^{14} labeled L-valine, L-phenylalanine, L-methionine
DNCl	15	1320	−45	960	−63	
0	—	2330		2000		3 hr prior to addition of C^{14} methyl L-methionine
DNBSO$_3$	7250	2510	+9	2170	+9	
DNP lysine	3000	2000	−15	1840	−8	

* For abbreviations see footnote on page 478

† *Sensitive donors* were injected 2 to 6 weeks previously with DNFB in Freund's adjuvant (0.2 μmole DNFB and 16 μg *M. butyricum* per animal). Subsequent skin testing with 0.01 M DNCB indicated a high degree of contact skin sensitivity. *Control donors* were injected 2 to 6 weeks previously with Freund's adjuvant alone (16 μg *M. butyricum* per animal).

‡ All incubations at 37° C. The number of cells per tube ranged from 2 to 5 × 10^7 and incubation time after addition of labeled amino acid varied from 30 to 60 minutes. In any given experiment equivalent numbers of cells from sensitized and control donors were treated identically. Values are averages of two to five replicate tubes and represent initial rates of incorporation.

pads at the same time with complete Freund's adjuvant alone. When the former had a high degree of DNP specific contact skin sensitivity, both sensitive and nonsensitive animals were injected into the footpads with DNFB in saline. Additional animals of both types were spared this second injection. Twenty-four hours later cells collected from the regional lymph nodes were assayed for rate of C^{14} labeled amino acid incorporation into cell protein. Cells from the four different kinds of donors all had about the same rate of incorporation (Table II).

DEOXYRIBONUCLEASE EFFECT ON ISOLATED CELLS

When isolated intact lymph node cells are incubated with deoxyribonuclease (DNase) for one hour the cells lose, maximally, 20 to 30 per

TABLE II AMINO ACID INCORPORATION INTO PROTEIN BY LYMPH NODE CELLS ISOLATED 24 HOURS AFTER INJECTION OF SENSITIZER *

First Injection	Second Injection	Rate of Amino Acid Incorporation cpm/mg protein/minute
DNFB in Freund's adj	DNFB in 0.15 M NaCl	33
DNFB in Freund's adj	None	37
Freund's adjuvant	DNFB in 0.15 M NaCl	35
Freund's adjuvant	None	30

For quantities of DNFB and mycobacteria injected in Freund's adjuvant see footnote of Table I. Seven weeks after the first injections 10 μg DNFB was reinjected and 24 hours later cells were collected. Eight days after the first injections, animals that had received DNFB were highly sensitive to 0.01 M DNCB. Cells were incubated for 30 minutes with 39 000 cpm C^{14} L valine (4800 cpm per mμmole valine) in a total volume of 2.0 ml. No C^{12} valine was added. The values given are means for quadruplicate tubes. Protein content of the isolated cell protein fraction was estimated spectrophotometrically at 280 mμ (solvent 0.25 M acetic acid; lymph node cell protein under these conditions has an extinction coefficient E_1^1 of 13.6)

cent of their DNA. DNase attacks "naked nuclei" prepared from these cells much more effectively and removes 90 per cent of their DNA over the same period.[16] Presumably DNase has less access to nuclear sites of intact cells than of "naked nuclei", and it is possible that if cells were "damaged" by exposure to an homologous sensitizer they might become abnormally susceptible to the action of DNase. Accordingly we have determined the extent to which DNase removes DNA from cells of sensitized and of nonsensitized donors following incubation with a DNP sensitizer. DNase removed the same amount of DNA from both kinds of cells after the cells had been incubated for 3 hours with 1.5×10^{-5} M DNCB (Table III).

STEADY STATE DISTRIBUTION OF SUGARS

The rate of entry of many metabolites into cells and their steady state distribution between extracellular and intracellular water reflect functions of metabolism and permeability which are dependent on cellular integrity. In looking for possible alterations in lymph node cell function it is therefore of interest to consider permeability for some simple sugars.

Distribution equilibrium of simple sugars between extracellular medium and isolated lymph node cells is reached too rapidly, even at low temperatures, to permit measurement of rates of entry into cells. Accordingly steady state measurements have been made and related, where possible

TABLE III DEOXYRIBONUCLEASE EFFECT ON CELLS INCUBATED WITH DINITROCHLOROBENZENE ‖

Donors*	DNCB†	DNase‡	DNA§	DNA Removed
			μg	per cent
Sensitive	0	0	235	31
	+	+	163	
Control	0	0	294	31
	+	+	203	

* *Sensitive donors* had a high degree contact skin sensitivity to DNCB induced by injection of DNFB in Freund's adjuvant 35 days prior to the experiment They were reinjected with DNFB in saline (10 μg per animal) 3 days before cells were collected Cells prepared in this fashion are ordinarily competent to transfer contact skin sensitivity 4 × 10⁷ cells per vessel *Control donors* were injected with Freund's adjuvant 34 days prior to the experiment 4 2 × 10⁷ cells per vessel

† 2 4 Dinitrochlorobenzene 1 5 × 10 M initial concentration

‡ 100 μg crystallized deoxyribonuclease (Worthington)

§ Deoxyribonucleic acid measured by diphenylamine method

‖ Tubes with DNCB were incubated 3 25 hours DNase was then added and the tubes were incubated for an additional 45 minutes Reference tubes were incubated for 4 hours without DNCB or DNase All tubes were in duplicate Incubation was terminated with cold 5 per cent trichloroacetic acid (TCA) Cold TCA insoluble material was extracted with warm ethanol ether (3 1) and then with hot 5 per cent TCA (95 C) The latter extracts were analyzed in duplicate for DNA Incubation temperature 37 C

to metabolic activities [15] The extent to which these sugars enter the cells has been expressed as a space which corresponds to the percentage of cell volume which is occupied by a solute at a concentration equal to its concentration in extracellular water A space is calculated as

$$\frac{a - (0\ 49cv)}{cv(1 - 0\ 49)} \times 100$$

where a is total amount of solute recovered from a cell pellet of volume v at an extracellular medium concentration of solute c Cell pellets are formed by centrifugation at 1300 × g for 5 minutes The correction factor 0 49 is that fraction of the packed cell volume which is extracellular fluid This value was obtained by measurement of inulin and raffinose spaces and since it is unexpectedly high confirmation by independent means has been sought Since we find an average of 6 × 10⁸ cells in 100 μl of packed cells the mean cell volume is 196 μ³ assuming the extracellular volume in packed pellets to be 49 per cent (i e, inulin and raffinose spaces) The value for mean cell volume corresponds for spherical cells, to a mean cell diameter of 7 2 μ Direct measurement of mean

cell diameter in the phase contrast microscope yields in good agreement the value $7.16 \pm 0.11 \mu$[13] *

As shown in Table IV cells from donors previously injected with

TABLE IV STEADY STATE DISTRIBUTION OF D GLUCOSE IN ISOLATED LYMPH NODE CELLS

Cells from Donors Injected with*	Number of Experiments	Glucose Space† (Mean ± S E M)	P Value‡
Nothing	14	26 ± 1 7	
DNFB in Freund s adjuvant	14	40 ± 2 7	<0 001
Freund s adjuvant	6	42 ± 2 4	<0 001

* For details of donor injection see footnote of Table I

† Values are percentage of packed cell volume occupied by gluco e at a concentration equal to its con entration in extracellular medium The values are corrected for extracellular space in cell pellets (49 per cent of pellet volume) S E M is standard error of mean

‡ Relative to cells from uninjected controls

DNFB in Freund s adjuvant or with Freund s adjuvant alone have a much larger glucose space than do the cells from uninjected control donors It may be noted that isolated rapidly growing Ascites tumor cells were found by Crane Field and Cori[8] to have a glucose space so high that the glucose concentration in total intracellular water was equal to extracellular glucose concentration If the latter condition applied to the cells used in the present work they would have a glucose space of 78 per cent We wonder therefore whether the increased glucose space in cells from sensitized donors is related to the rapid growth of stimulated lymph nodes with an increase in the number of immature and rapidly proliferating cells

RESPIRATION AND GLYCOLYSIS

Isolated lymph node cells behave quantitatively like many normal metabolically active tissues[15] Without the addition of glucose or other substrate the cells consume oxygen vigorously but produce essentially no lactic acid When glucose is added to the incubation medium lactic acid production is low in the presence of oxygen but rises about fourfold

These calculations apply to cells from lymph nodes of guinea pigs injected with Freund s complete adjuvant (4 μg *Mycobacterium butyricum* per footpad with or without the addition of other antigens) In the case of lymph node cells from uninjected guinea pigs the number of cells per 100 μl packed pellet is closer to 3×10 which corresponds to an extracellular volume of about 41 per cent with a mean diameter of 7.2μ The inulin space in pellets of cells from uninjected animals is 45 per cent The data of Table IV were all calculated on the basis of a 49 per cent extracellular volume If a 45 per cent value were used for the cells from nonsensitized animals the differences shown in the table would be slightly less but still significant

under anaerobic conditions Cells obtained from uninjected donors and from donors injected with a variety of sensitizers with or without Freund's adjuvant behave indistinguishably as regards these parameters (Table V)

During the ten days following injection of sensitizer in Freund's adjuvant the regional lymph nodes increase in weight almost tenfold Never

TABLE V RESPIRATION AND GLYCOLYSIS OF ISOLATED GUINEA PIG LYMPH NODE CELLS*

Cell Donors†	No of Experiment	Added to Medium		$-QO_2$	$+QCO_2$	$+Q^{N_2}_{Lactate}$ ‖		$+Q^{O_2}_{Lactate}$
		Amino Acids‡	Glucose§			Manomet	Chemically	
Control	4	−	−	13	13	0 4	0 5	
	4	−	+	16	19	2 1	2 7	
	1	+	−	25	23	—	—	
	2	+	+	27	30	2 4	2 5	
A	5	−	−	14	14	0 4	0 2	0 6
	8	−	+	12	15	2 2	2 5	9 5††
B	4	−	+	12	13	1 0	2 2	
C	2	−	+	15	17	2	2 6	
D	4	+	−	25	26	1 4	0 4	
	4	+	+	21	24	3 2	3	
E	4	+	−	25	27	2	0 4	0 7
	4	+	+	20	23	3	3	11 0††

* Results are mean Q values expressed as µl mg dry weight cells for first hour at 37 C Gas phase 95 per cent O_2 − 5 per cent CO_2 *except* as noted in footnote All aerobic measurements made with Warburg's two-vessel method Coefficient of variation for QO_2 and QCO_2 average 12 and 15 per cent respectively and for $Q_{Lactate}$ (chemically analyzed) was 22 per cent

† Control uninjected normal animals A Injected with DNFB in Freund's adjuvant 3 to 6 weeks previously *B* Like *A* but reinjected with DNFB 24 hours before the experiment These cells are ordinarily competent to transfer contact skin sensitivity *C* Injected with Freund's adjuvant alone 3 to 6 weeks previously injected with DNFB 24 hours before experiment — hence a control for group *B* *D* Injected with DNP beef gamma globulin in Freund's adjuvant 6 weeks before *E* Like *D* but reinjected with DNP beef gamma globulin 42 hours before

‡ The 13 essential amino acids of Eagle's cell culture medium (*Science* 122 501 1955)

§ 5.5×10^{-3} M

‖ Lactate analyzed manometrically assuming an R Q of 1 0 and also chemically Gas phase 95 per cent Argon − 5 per cent CO_2

†† Meyerhof quotient $\left[\frac{Q^{N_2}_{Lactate} - Q^{O_2}_{Lactate}}{QO_2}\right]$ averages 0 6

theless cells isolated from such rapidly growing nodes differ from isolated Ascites tumor cells in a number of respects Firstly the lymph node cells exhibit a pronounced Pasteur effect that is they show about a fourfold inhibition of glucose utilization and of lactic acid production by O Secondly, in spite of the lymph node cells having high endogenous respiration they do not demonstrate a Crabtree effect — that is their oxygen consumption is not inhibited when glucose is added and utilized correspondingly their Meyerhof quotient is below 1 (see Table V) For neoplastic cells a value greater than 1 is usually found Finally as mentioned above glucose space is appreciably lower in stimulated lymph node cells than in the tumor cells (41 per cent as compared with 78 per cent)

In a few experiments animals previously injected in footpads with DNFB or DNP protein in Freund s adjuvant and control animals previously injected with Freund s adjuvant alone were reinjected with the same sensitizers in the same footpads 1 to 2 days before cells were collected from lymph nodes regional to the injection sites This procedure may be regarded as an *in vivo* challenge of lymph node cells by homologous sensitizers As shown in Table V cells isolated from a variety of reinjected donors and from appropriate control animals did not differ as regards oxygen consumption and glycolysis

Comments

In respect to the two questions asked initially the experimental results presented may be summarized as follows

(1) Cells isolated from antigenically stimulated lymph nodes of guinea pigs with a high degree of DNP specific contact skin sensitivity behave no differently than the corresponding cells of DNP insensitive animals when incubated with DNP sensitizers This is true even for cells which are competent to transmit DNP specific contact skin sensitivity to a normal recipient

(2) Cells isolated from lymph nodes regional to injection of sensitizers in Freund s adjuvant or to injection of Freund s adjuvant alone differ from the lymph node cells of uninjected control animals in respect to their accommodating more intracellular free glucose This difference is not restricted to a metabolizable sugar since we have observed consistently in several experiments the same difference with D arabinose a pentose which is presumably not utilized by mammalian cells Previously we raised the question whether the increased glucose space might be related to increased cell proliferation in intensely stimulated lymph nodes It is pertinent to note in this connection that spleen explants from guinea pigs previously injected with tubercle bacilli have been observed to proliferate

much more actively than spleen explants from uninjected normal animals The demonstration of a definite functional distinction between lymph node cells of unstimulated donors and of intensely stimulated donors underscores the problem involved in the choice of controls for study of specific cytotoxic effects on isolated cells and tissues An essential requirement for control purposes are donors that have been sensitized with antigens which are physiologically equivalent but immunologically unrelated to the antigen under study Uninjected normal controls which have been used almost exclusively in the past as the sole source of control tissue in tissue culture studies are insufficient for demonstrating specificity of response (for exceptions see references 19–20)

Many possibilities could account for the failure to observe specific metabolic changes following addition of homologous sensitizers to cells obtained from donors with a high degree of contact skin sensitivity It is certainly possible for example that the intensity of hypersensitivity induced was not as great as that sometimes attained with antigens of bacterial origin We wish to discuss chiefly however the possibility that cells obtained by the teasing procedure from lymph nodes are excessively heterogeneous in respect to immunologic function If only a small proportion of the cells in these populations are specifically reactive any altered response to sensitizer they might exhibit would very likely be obscured by the nonspecific response of the majority of cells which are in this sense contaminants

In respect to antibody formation in the rabbit there is impressive evidence that perhaps only 1 to 10 per cent of cells in lymph nodes regional to footpad injections of antigen contain or make antibodies[6 7 18] Since contact skin sensitivity is not dependent on antibody at least as it occurs in serum extrapolation from antibody localization in the rabbit to delayed type hypersensitivity in the guinea pig carries much uncertainty Nevertheless it would not be surprising if in the teased out lymph node cell populations used in our study only a small fraction of the cells should turn out to be specifically functional in respect to contact skin sensitivity

It must be emphasized that the heterogeneity discussed above refers to immunologic function not to morphology Actually the cell populations used in the present work are fairly homogeneous anatomically By phase contrast microscopy and by examination of stained smears they appear as a rule to be about 80 to 85 per cent small lymphocytes 10 per cent large mononuclear cells and 5 per cent erythrocytes In an electron micrograph kindly prepared and interpreted by Dr Sam Clark Jr of the Department of Anatomy a typical preparation revealed a greater degree of morphological heterogeneity but even by this procedure perhaps as many as 75 per cent of the cells appeared to be small lymphocytes Since we are considering functional heterogeneity at a level where

the cells of interest might comprise only 1 to 10 per cent of the total (conceivably much less), it is plain that experiments which deal with mixed cell populations can hardly make useful correlations between functional and anatomical cell type

Are there populations which might contain a greater proportion of specifically reactive cells? In tissue culture experiments in which explants were described as being specifically injured by homologous antigens tissues were derived from many sources in addition to lymph nodes—for example spleen, bone marrow and buffy coat of blood (Migratory macrophages in particular have been noted to be especially altered in tissue culture demonstrations of cytotoxic responses[14, 22]—whether primarily, due to action of antigens on these cells, or secondarily, due to antigen interaction with immobile cells within the explant is not clear) Moreover in the first demonstration of transfer of contact skin sensitivity Landsteiner and Chase used cells from peritoneal exudates[17] Indeed one of the most strikingly positive cell transfer experiments carried out in this laboratory was accomplished not with the cells teased from lymph nodes but with residual lymph node pulp (remaining after teasing) which was simply placed in the peritoneal cavity of a recipient guinea pig By a simple technique which involves slow stirring of lymph node pulp residues that remain after teasing out of cells Cohn and Attardi have obtained populations which have sometimes a higher frequency of antibody forming cells than do populations which are teased from the nodes[8] In one experiment using their technique we have observed transfer of contact skin sensitivity with several fold fewer pulp cells than was possible with cells teased from the very same lymph nodes The data are shown in Table VI It is therefore entirely possible that there exist cell populations with a higher specific activity in respect to contact skin sensitivity than cells teased from lymph nodes

Many years ago Dienes suggested that delayed type hypersensitivity occurs consistently soon after injection of protein antigens and before serum antibody is detectable[10] On the basis of new evidence Pappenheimer has made a similar suggestion and has drawn provocative parallels with certain phases in induced enzyme formation in bacteria (see Chapter 25 of this volume) It is appropriate in this connection to refer briefly to recent observations in this laboratory concerning phosphoprotein synthesis by isolated lymph node cells[9] When P^{32} orthophosphate is incubated with the cells P^{32} is incorporated at a linear rate into a protein fraction which is isolated in the same way as, and has the same properties as the protein fraction described above in connection with the C^{14} labeled amino acids Acid hydrolysis of this fraction has moreover yielded P^{32} O phosphoserine Of particular significance for the present discussion is the fact that the rate of P^{32} incorporation into phosphoprotein is enhanced by

TABLE VI TRANSFER OF CONTACT SKIN SENSITIVITY WITH LYMPH NODE PULP CELLS

Type of Lymph Node Cells	Number of Cells Injected into One Recipient	Skin Test Response of Recipients
	×10⁸	
Teased	9 0	2+
	4 5	±
	1 9	0
Pulp	1 5	3+

Four donor guinea pigs were injected with DNFB in Freund s adjuvant each animal receiving a total of 0 1 µmole DNFB and 80 µg *M butyricum* Seventeen days later they were injected again in the footpads with DNFB in 0 15 M NaCl each animal receiving 10 µg DNFB Three days after the second injection axillary popliteal and inguinal lymph nodes of all donors were combined and cells were teased out The residual lymph node pulps were stirred slowly at about 4 C for 1 hour [6] the cell obtained by this process are designated pulp cells Cells were injected intraperitoneally Two days after transfer recipients were skin tested with 0 01 M DNCB in 1 1 corn oil acetone

antigenic stimulation Lymph node cells from animals injected with a protein in incomplete Freund s adjuvant were compared with control cells obtained from donors injected with incomplete Freund s adjuvant alone Four days after footpad injections the rate of incorporation of P^{32} into phosphoproteins is 30 per cent greater in cells from protein injected donors than in cells from control injected donors The difference between the two is transient it is not observed on the third day after injection and is inconstant on the sixth day The significance of the phosphoproteins made by these cells is not known They do not appear merely to serve as a source of rapidly turning over phosphate since addition of excess P^{32} orthophosphate at any time during incubation immediately blocks further incorporation of P^{32} but does not displace P^{32} previously incorporated

If we suppose that delayed type hypersensitivity is characterized by antibodies which are bound in the cells that form them we are entitled to speculate about the bonds that could account for intracellular immobilization of these molecules Disulfide and amide bonds and of course many others also could serve to bind antibodies to fixed cell structures There are two observations however which lead us to speculate that phosphodiester bonds in particular might be adapted for sequestration of antibody in intracellular sites (1) antigenic stimulation results in enhanced phosphoprotein synthesis and ( ) threonine and serine together comprise about 20 per cent by weight of gamma globulins The latter

amino acids have side chain hydroxyl groups and are readily phosphorylated Phosphodiester bonds need not modify the configuration of the binding sites of antibodies and specific interactions might therefore be possible for antibodies immobilized within cells through phosphodiester bonds which tie them to fixed cell structures It is tempting to conjecture further that the splitting of such bonds by appropriate phosphodiesterases could result in liberating antibody so that it is free to move into extracellular water Most of what we know or think we know about delayed type hypersensitivity is consistent with this speculation However very few facts concerning this phenomenon are clearly established and it is evident therefore that many other speculations equally consistent could be considered

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30

Histamine in Allergic Responses of the Skin

THEODOR INDERBITZIN M D

(Zurich Switzerland)

There are two types of allergic reactions the immediate type (typified by cutaneous anaphylaxis and Arthus phenomenon) and the delayed type (typified by tuberculin and contact dermatitis type of reaction) It is firmly established that histamine is released from the skin in the course of allergic reaction of the immediate type In contrast to this the delayed type *of reaction is usually considered to be independent of the release* of histamine This view is based upon the observation that this type of skin response shows an inflammatory process rather than vascular changes and certainly intradermal injected histamine will not duplicate the changes of the delayed response Figure 1 shows the histamine levels of the skin at the site of three different allergic reactions In the cutaneous anaphylaxis and in the Arthus phenomenon the injection of antigen induces a rapid decrease of the histamine content of the skin In the Arthus phenomenon the release of histamine is followed by an accumulation of histamine which coincides with a cellular infiltration of the site mainly by polymorphonuclear leukocytes

While it is generally believed that histamine plays no part in the delayed type of sensitivity I have found a histamine increase in tuberculin reaction and contact dermatitis No initial histamine depletion could be detected as in the immediate type of reaction A steady rise of histamine content begins 3 hours after the challenge, and a maximum is reached in the tuberculin reaction after 7 hours This histamine rise is similar to that which occurs in the second phase of the Arthus phenomenon and coincides also with the inflammatory dermal infiltration by mononuclear cells

The obvious coincidence between the cellular infiltration and the histamine accumulation leads to the assumption that the histamine is imported by the infiltrating cells This idea is supported by the fact that the white blood cells carry preformed histamine and especially by the fact that the histamine levels depend on the degree of the reaction In the tuberculin reaction the histamine increase runs parallel with the lesion diameter (Figure 2) Cortisone depresses both the histamine accumulation and the

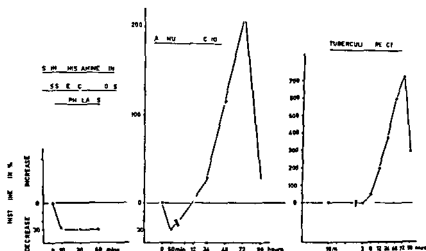


FIGURE 1 Histamine content of skin in passive cutaneous anaphylaxis Arthus phenomenon and tuberculin reaction

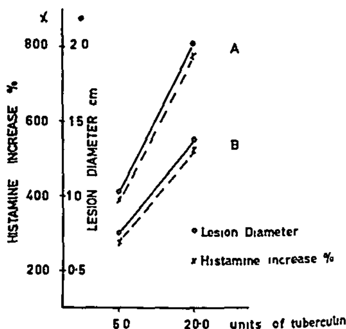


FIGURE Histamine content of skin in the tuberculin reaction

inflammatory infiltration. When an experimental lymphopenia of tuberculin sensitive animals was induced by a rabbit anti lymphocyte guinea pig immune serum neither an allergic lesion nor an increase of histamine was detected after the specific challenge. In contrast to this a polymorphonuclear penia induced by specific antiserum does not markedly alter the allergic response or the histamine accumulation. A depression of tuberculin reaction and of histamine levels seems due to immune cross reaction and the partial lymphopenia induced by the antipolymorphonuclear serum (Figure 3)

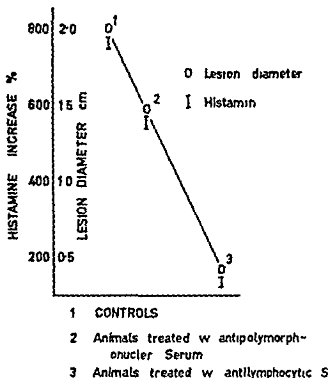


FIGURE 3 Effect of antileukocytic sera on histamine content of skin

These facts demonstrate the importance of the lymphocytes as sensitivity carriers and as carriers of biological substances

Two questions arise. Is the histamine accumulation observed in these allergic reactions specific and if so what are the functions of the released histamine and of the accumulated histamine?

Neither the release of histamine nor the accumulation of histamine is a specific process seen only in allergic response. Figure 4 illustrates the changes of histamine levels after freezing of the skin with chlorethyl

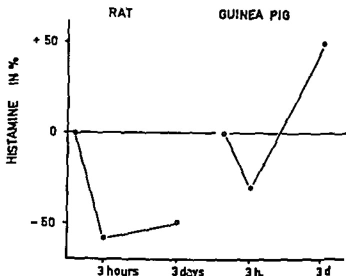


FIGURE 4 Skin histamine after refrigeration

The skin damage leads as in the Arthus phenomenon first to a depletion of the skin histamine and then with the onset of the inflammatory reaction to an increase of histamine In rats the normal skin histamine level is very high whereas in guinea pigs it is low In rat skin the amount of histamine depletion by freezing is not compensated by the following inflammation whereas in guinea pigs the depletion of histamine is followed by a histamine increase coinciding with the cellular infiltration of the lesions There are thus two factors determining the amount of histamine present in an inflammatory lesion of the skin (1) histamine liberated from the skin in response to various insults and (2) histamine transported by infiltrating cells to the site of the inflammation However it seems that the amount of histamine measured at the sites of Arthus reaction and the delayed type of allergic reactions cannot be explained by the import of histamine by the white blood cells only The histamine accumulation in these reactions seem to exceed the amounts of histamine carried by the circulating lymphocytes or polymorphonuclears

Histamine liberation as well as histamine increase associated with a polymorphonuclear infiltration can easily be reproduced by a number of unspecific skin conditions The excessive histamine accumulation simultaneous with lymphocytic infiltration characteristic of the delayed type of skin reaction cannot be reproduced by a nonspecific challenge and points out the importance of the lymphocytes in this type of reaction

In the immediate type of allergic reaction there is release of histamine following antigen antibody combination Since lymphocytes bring antibody to the site of delayed allergic reaction histamine liberation could

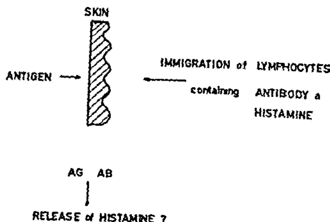


FIGURE 5 Hypothetical explanation of histamine release in delayed type sensitivity

be expected Application of antigen (Figure 5) to the skin induces an immigration of sensitive lymphocytes from the blood stream This process takes several hours It explains the delay of the beginning of the reaction Thereafter the antigen antibody reaction may lead to liberation of histamine from the lymphocytes giving rise to the long lasting vascular dilatation and increased permeability

There is a difference between the histamine depletion in the cutaneous anaphylaxis and the Arthus phenomenon and that shown after an unspecific traumatization of the skin Our experiments show that an allergic reaction always liberates the same amount of histamine from areas of skin identical in size The amount of histamine released is fully independent of the amount of reacting antigen antibody In other words in passive cutaneous anaphylaxis induced with o gamma antibody N no greater histamine depletion was detected than in the first stage of the reversed Arthus phenomenon induced with 1000 gamma antibody N The liberated histamine in both reactions amounts to 30 per cent of the total histamine content We found the same values for urticarial reactions in human skin and anaphylactic reactions in the guinea pig and rat skin

The histamine depletion at the site of immediate allergic reactions differs fundamentally from that induced by traumatization such as freezing or by injection of histamine releasing compounds With these unspecific reactions the amount of histamine liberated depends upon the degree of traumatization Only intradermal injection of proteolytic enzymes will release histamine in amounts comparable with those found in skin anaphylaxis (Figure 6) This fact emphasizes the question whether the vascular effects seen in immediate allergic skin reactions are responses to

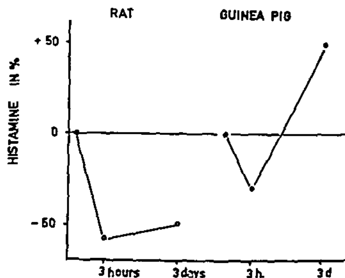


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mine seems to be responsible for the observed vascular or inflammatory changes Its pathophysiological function is not yet understood

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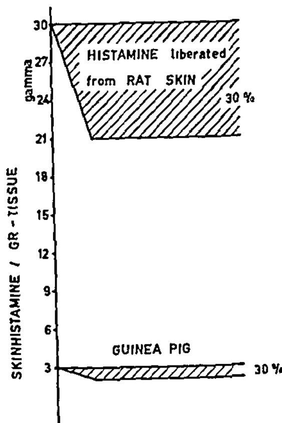


FIGURE 6 Histamine liberated by proteolytic enzymes or anaphylaxis

released histamine As stated above only proteolytic enzymes injected intradermally release histamine from the skin in amounts comparable to those seen in cutaneous anaphylaxis Proteolytic enzymes induce vaso dilatation and an increase on capillary permeability as well as polymor phonuclear cellular infiltration all of which occur in the anaphylactic type of skin reaction The antihistamines are also without much effect. The vascular changes and inflammation produced by proteolysis are com parable with the vascular reaction and inflammation resulting from cu taneous anaphylaxis Yet to date there is no evidence that proteolytic enzymes play any part in skin anaphylaxis But if such evidence would be forthcoming it would indicate that the histamine release is merely a side effect accompanying a chain of events following antigen antibody reaction

As for the kind of mechanism responsible for the vascular and inflam matory changes in the delayed type of reaction, we do not even have a hypothesis All we can say is that in both types of allergic skin reactions, immediate and delayed neither the liberated nor the accumulated hista

delayed allergy That this might be due to the minute amounts of gamma globulin which they possess is a possibility but we consider it remote

RUSSELL S WEISER (Seattle Washington) I want to direct my comment to Dr Salvin

Some years ago we went over much of the same ground that Dr Salvin has gone over using bovine gamma globulin in very small amounts injected into the skin We noted essentially the same results It was our interpretation or at least one of the possible interpretations that when a skin reaction is first obtained on about the fourth or fifth day after the initial injection the delay in the onset time of the reaction represents an anamnestic response that is it represents the time necessary for the formation of more antibody sufficient to produce the reaction

This was the explanation that we thought of at the time and I should like to introduce this concept for whatever it is worth We never were fully convinced that these were truly delayed tuberculin type reactions because we could never get corneal reactions and we could never produce systemic reactions that looked like the tuberculin reaction I might say that in animals that were retested and stimulated we did observe the rings in the eyes which Dr Germuth has described

BRAM ROSE (Montreal, Canada) I should like to ask a question of Dr Good and also one of Dr Inderbitzin

Dr Good in his description of his investigations made no mention at any time of whether or not these children with agamma globulinemia have eosinophils I wonder if he would care to comment on the eosinophilia – if it exists in these children and if there is any correlation with any of the findings he has described

With reference to Dr Inderbitzin s work This is an extremely interesting presentation and it is a rather crucial one with reference to the role that histamine may play and the effect of various agents such as the antihistamines or the steroids

The immediate skin reaction which is undoubtedly due to the release of histamine, as he has said (and with which most people agree) is not affected by steroids but can be abolished by antihistamines The Arthus phenomenon and the tuberculin reaction are both amenable to the activity of the steroids and on the other hand are apparently unaffected by the administration of antihistamines

I wonder if Dr Inderbitzin has carried out an experiment by means of which he has simply induced an inflammatory response by some other means and has observed the accumulation of histamine in such a lesion It could mean simply that histamine is brought there by cells that play no significant role although I am sure Dr Schild will arise and say that they

GENERAL DISCUSSION

WILLIAM J KUHNS (Pittsburgh Pennsylvania) I should like to ask Dr Good a question

Dr Good, I noticed on your slide and in your talk that considerable individual variability exists in the so called agamma globulinemic group in regard to the ability to develop a delayed type hypersensitivity I wonder if this would have any relation to whether the person has hypogamma globulinemia or whether he has complete agamma globulinemia as determined by immunologic means Is there any evidence that minimal quantities of gamma globulin are required in order for the delayed type of hypersensitivity to be elicited

On the other hand do your studies indicate that persons completely devoid of gamma globulin as demonstrated by all available methods cannot produce the delayed form of reaction?

DR GOOD This point has troubled us a good deal We must conclude in interpreting our own data that truly agamma globulinemic patients do not exist I am fully aware that the possibility exists that what we are measuring and calling tiny amounts of gamma globulin may in reality represent cross reactions with other serum protein However we think this is not the case, and are convinced that we are really measuring minute amounts of gamma globulin

These antisera have been prepared against highly purified gamma globulin in addition we have done very careful absorption of the anti serum with agamma globulinemic sera to remove contaminating antibodies Further there is an internal consistency in that the patients with the largest amounts of gamma globulin are actually able to form tiny amounts of antibody When we find gamma globulin values of 40 to 100 mg per cent we have been able to stimulate the patients to form a small amount of antibody in several cases

We have not seen in the agamma globulinemic group any patients who would not develop delayed allergy after appropriate stimulation The patients I presented who did not develop delayed allergy were immunologically normal children and I am not sure they did not go through a phase of delayed allergy only to lose this as they began to form circulating antibody We cannot evaluate this point because we did not perform serial skin tests on them All our results were obtained by skin testing 12 to 14 days after application of the antigen or specific immunologic precipitate Thus Dr Kuhns I don t think we can answer your question in a very critical way We think all of the patients with hypogamma globulinemic syndrome whom we have studied have been able to develop

delayed allergy That this might be due to the minute amounts of gamma globulin which they possess is a possibility but we consider it remote

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do I am merely asking for information and am not making a statement

Dr Good The point about the eosinophils has interested us a good bit Most patients with hypogamma globulinemia have eosinophils in the bone marrow and they may develop some eosinophilia of the circulation and bone marrow during the recovery phase from infection However we have studied one patient an adult with acquired hypogamma globulinemia who was completely lacking in eosinophils in the circulating blood bone marrow and tissues

The little child whom I presented on the slide having the skin disease I referred to as atopic type dermatitis did not have a marked eosinophilia He had 430 eosinophils per cubic millimeter of blood by specific count

There was one question that was obvious in my own presentation which I want to comment on before I close Is there any explanation for the fact that these agamma globulinemic patients do not develop delayed allergy to streptococcal and pneumococcal products This certainly has been very consistent we have not seen delayed reactions to streptococcal and pneumococcal antigens in any of the 15 cases of agamma globulinemia that we have studied in this way

I think certain observations on the streptococcal and pneumococcal infections in these patients probably explain what has occurred If we do not treat these infections early in their course and get rid of the microorganisms the infections extend and produce generalized disease In other words we have to eliminate these microorganisms from these patients in order to make it possible for them to survive

Thus I believe the failure to develop delayed allergy to these antigens is a consequence of the treatment and elimination of the organism in these patients and that it probably is not due to any intrinsic defect in the mechanism for development of delayed allergy

Dr Inderbitzin As I mentioned the amount of histamine present in an inflammatory lesion of the skin depends upon two factors the histamine liberated from the skin and/or the histamine transported by infiltrating cells to the site of the inflammation

We found an increase in the local histamine content in various unspecific (nonallergic) skin inflammations Histamine release as well as histamine increase in the course of an inflammatory skin process varies fundamentally according to the species under consideration and the type of reaction The local increase of histamine in the reacting areas during delayed skin allergies is dependent upon the availability of lymphocytes but is not necessarily the result of the histamine contained in these infiltrating cells

J Prochazka Fisher (New York New York) Dr Inderbitzin has presented a summary of his studies of histamine levels at the site of tuberculin and eczematous reactions He calls particular attention to three questions (1) Are histamine levels specific for these allergic reactions? ( ) What is the source of the histamine? (3) What is its role At the Institute of Allergy Roosevelt Hospital, New York City during the past two years Dr Robert A Cooke and I have made chemical determinations of histamine in primary toxic and in allergic contact dermatitis of guinea pigs I shall briefly describe some of our results as they afford possible answers to the question Dr Inderbitzin has raised I shall discuss them in the order mentioned

At the sites of allergic contact dermatitis of sensitized guinea pigs after epicutaneous application of 0 1 per cent 4 dinitrochlorbenzene (referred to as DNCB) in olive oil, a steady rise in histamine begins after the challenge The maximum is reached at 48 hours It is more than 400 per cent above the average amount measured in normal skin at the sites of olive oil controls

A single application of . per cent DNCB induced a primary toxic dermatitis in all the animals tested however the changes in histamine content in these lesions are not significant In the primary toxic lesions induced by 5 per cent DNCB the histamine content rises and reaches a maximum in 48 to 7 hours It is only 80 per cent above the amount of histamine in normal skin On the third fourth and fifth days these toxic lesions regress and the histamine content falls On the sixth day and thereafter, lesions begin to flare up The histamine reaches a new peak about the ninth day It is over 300 per cent above the histamine content of normal skin The histological changes in the regenerated epidermis underneath the slough of the toxic reactions now have the histological characteristics of allergic contact dermatitis Epicutaneous testing on a new area of the skin detected sensitivity in some animals by the seventh day and in all by the eleventh day Apparently a high histamine level is associated with the allergic dermatitis but not with the primary toxic dermatitis in normal animals before sensitization develop

To discern the probable source of the histamine accumulating at sites of allergic dermatitis we have first determined the histamine content of the whole blood of sensitized guinea pigs We found that the histamine content in allergic lesions induced by 0 1 per cent DNCB was too high to have been supplied by the infiltrating leukocytes alone The histamine increase per 1 Gm of skin would require import of all histamine from 19 to 25 ml of whole blood – more blood than the 500 Gm guinea pig has We determined the histamine content in allergic lesions induced by 0 01 per cent DNCB and made counts of the infiltrating cells in histological sections The histamine content of these allergic lesions was more

than twice as much as that measured at the sites of primary toxic dermatitis induced in normal animals by 5 per cent DNCB, the number of infiltrating cells on the other hand was far lower *These observations led us to the assumption that at the site of allergic dermatitis an activation of histamine metabolism is responsible for the high histamine level*

To test this assumption we made two experiments Allergic dermatitis was induced in two groups of guinea pigs and the histamine levels determined Fifteen weeks later one group was injected with Germanine (Naphuride sodium brand of seramine sodium Winthrop Stearns Inc was reported as a decarboxylase inhibitor preventing formation of histamine from histidine) and the other with *l* histidine While being injected each group was retested and histamine levels again established Far less histamine was found in the lesions of the animals injected with Germanine whereas the histamine had increased in the lesions of animals injected with *l* histidine However in the latter group a histamine increase was found throughout the integument It seems that the Germanine has interfered with decarboxylation This supports the assumption that the histamine accumulation in allergic dermatitis is brought on by activation of histamine metabolism The fact that administration of *l* histidine did not promote a far greater increase of histamine in the allergic lesion than in the integument does not necessarily weaken this assumption It might merely indicate that the decarboxylase activity alone is responsible for histamine increase and not the amount of *l* histidine introduced into the metabolic process

Our findings do not fully explain the role of histamine in allergic dermatitis Allergic lesions in guinea pigs injected with Germanine were *of much longer duration than allergic lesions running their* usual course It seems in order to suggest that histamine is an accelerating factor in the repair process rather than a causal factor of the allergic dermatitis

VIII

Immunologic Unresponsiveness

Chairman FREDERICK G GERMUTH JR MD

(Baltimore, Maryland)

31

*Immunologic Unresponsiveness to Allergenic Chemicals**

MERRILL W CHASE PH D and JACK R BATTISTO PH D
(New York New York)

This presentation deals with a fortunate rediscovery made quite by chance around 1943 and with the studies subsequent to it † It turned out that guinea pigs which had been used as negative (normal) controls for tests being conducted on sensitized animals and had received a minimal amount of allergenic chemical applied to their skins resisted a later deliberate attempt to sensitize them so as to possess delayed type contact hypersensitivity to the same chemical In searching to reproduce the phenomenon at will, we finally hit upon an entirely different technique truly an artifice of the laboratory namely the *feeding* of allergenic chemicals before any attempt was made to induce sensitization

Only later did we realize that the same principle had been encountered previously by Dr Marion Sulzberger in his studies with neoarsphenamine [1] In Dr Sulzberger's cave animals received a single intravenous injection of neoarsphenamine and later were given a single sensitizing injection of the same compound When brought to test 4 weeks later the pretreated animals were found to have resisted the sensitization The observation that Dr Sulzberger had made could not be pursued readily for it proved almost impossible for him to sensitize guinea pigs to neoarsphenamine after he returned from Switzerland and in particular the establishment of inhibition by intravenous injection is so seldom possible that the method could not serve as prototype for general studies By chance our own observation provided a broad base that was capable of further extension

* Portions of this investigation were supported by a postdoctoral fellowship (GF 4487) from the National Institutes of Health U S Public Health Service to Dr Battisto whose present address is Albert Einstein College of Medicine Yeshiva University New York N Y The study was made at the Rockefeller Institute for Medical Research New York

† In addition to the experiments forming the basis of this paper [2 7 8] it should be mentioned that work is currently being conducted in collaboration with Dr Roy L Ritts For the portion of the more recent work quoted here acknowledgment is made of the support by a research grant (E 1258) from the National Institute of Allergy and Infectious Diseases U S Public Health Service

A third instance of immunological paralysis was discovered by Felton in 194 [1] and later studied by other workers [1, 17, 19, 20] Pneumococcal polysaccharide injected in proper amount apparently without requirement for selected routes of administration and in this respect unlike the instances cited above survives for a long time in the tissues and prevents the animal from responding to immunizing procedures

A fourth type of induced immune unresponsiveness (with respect to isoantigens thereby permitting tolerance of skin homografts [4]) is presented in Chapter 35 of this volume

Perhaps an additional type of immunologic unresponsiveness is that which can be established by administering protein antigens although it appears to be rather less durable This subject is reviewed by Dr Weigle in Chapter 33

The allergenic chemicals that have been fed to groups of guinea pigs or applied to them in special ways include 2 4 dinitrochlorobenzene picryl chloride *o* chlorobenzoyl chloride phthalic anhydride and citraconic anhydride

Several characteristics of the state of immunologic unresponsiveness are to be emphasized (*a*) The refractory effect is specific for the compound fed consonant with chemical cross relationships (*b*) Once established the resistance to undergo contact type sensitization is quite durable so far as can be told by careful observation the resistance first established remains undiminished for nine or more months but by the twelfth or thirteenth month one sees some lessening in degree of unresponsiveness when compared with prior tests on preceding members of the same inhibited group (*c*) The effect is not absolute deliberate experimental courses in sensitization applied to fed animals will incite weak vestiges of a positive allergy in the bulk of animals but even two or more further courses do not increase the degree of hypersensitivity (*d*) The unresponsiveness not only is found to be an inability to develop typical contact type hypersensitivity but represents a resistance to development of circulating antibody when proteins coupled with the same chemical grouping are injected (*e*) The tissues of the fed and resistant animal do not take up specific antibody when it is perfused into them. This finding is consistent with the fact that the unresponsiveness can be established by quite small amounts of specific allergen as we know from instances in which the state of unresponsiveness has arisen from skin painting and as we can judge when radiocarbon picryl chloride is fed in attempting to learn the amount that passes the gut wall (*f*) Feeding does not induce desensitization in sensitized animals

Our present working hypothesis is that the observed immunologic unresponsiveness is referable to a rather permanent coupling of a specific chemical grouping to living tissues forming a sort of immunologic

chimera in sites, perhaps intracellular ones that somehow control responses of the immunological apparatus If this view is correct the process would be related basically to that operating in the production of homograft tolerance by cells[4] and cell extracts (perhaps in the immunologic unresponsiveness to protein antigens as well[*]) when these materials are given in late fetal or neonatal life As in the other instances cited it is necessary to operate in ways that avoid early active stimulation of the immunological apparatus or only partial inhibitory effects will be secured

In our own work mature guinea pigs are used As for operational details it is necessary to avoid contamination of the skin particularly around the lips lest active sensitization result Because of the location of molar teeth it is difficult to feed gelatin capsules with dry chemical mixed with lactose More satisfactory is the feeding of solutions made in triglyceride oils (olive oil corn oil) While earlier feedings were made by pipette to guinea pigs suitably restrained and positioned more recently a much improved method of feeding using a fixed hypodermic syringe an adapter and a bit of infant's feeding tube has been developed by Dr Ritts Our patterns of feedings fall into two principal categories the amount fed varying somewhat with the chemical employed Typically we give 5 or 6 daily feedings of 3 mg dissolved in 0 3 ml of olive oil on each of 3 alternate weeks totaling 45 to 54 mg or 3 daily feedings of the same amount on each of 3 successive weeks totaling 27 mg In the case of 2 4 dinitrochlorobenzene one third the concentration given may suffice Thereafter the animals are rested for several weeks before attempts are made to induce active sensitization Such attempts commonly take the form of a series of 10 to 15 daily injections into the skin each of 2 5 µg of freshly prepared solutions in saline[8] Contact testing is conducted as previously described[8] usually with 2 or 3 concentrations of the chemical dissolved in an oil different from the one employed for the feedings, and with the oily vehicle itself as well

It is found that no animal is sensitive to any considerable degree while few are *entirely* negative This degree of inhibition readily permits laboratory study with modest numbers of animals (Figure 1)

The resistance established by feeding is specific for the compound fed (Table I) Animals were fed one compound either dinitrochlorobenzene or orthochlorobenzoyl chloride and were sensitized with both compounds by means of injections into the skin on alternate days After a rest period contact tests were made with both substances dissolved in olive oil—dinitrochlorobenzene (1 per cent ⅓ per cent) and orthochlorobenzoyl chloride (10 per cent 3 per cent) The difference in the dosage of the two chemicals reflects the high lability of orthochlorobenzoyl chloride to moisture, and its decomposition to orthochlorobenzoic acid One sees

* See Chapter 33 by Weigle and also reference 11

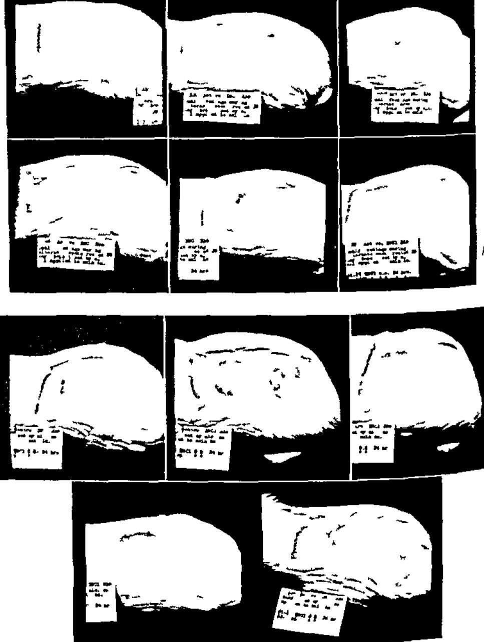


FIGURE 1 Induced resistance of sensitization with 2,4 dinitrochlorobenzene The 6 animals shown in A were fed a 1 per cent solution of dinitrochlorobenzene in corn oil (daily feedings of 0 3 ml for 6 successive days during the first third and fifth weeks) The 5 animals shown in B did not receive such feedings Twenty nine days after the final feeding all animals were subjected to painting with several drops of 2 per cent solution of dinitrochlorobenzene in absolute alcohol applied directly to skin at base of neck daily for 5 days. Seventeen days later contact tests were made with 1 drop of 1 per cent solution in olive oil (cephalad) and 1 drop of 1/3 per cent (caudad) Reactions photographed 24 hours later Reproductions from Kodachrome 35 mm film by Mr Julian Carlile Views taken with Elmar f/3 5 lens with Leica No 3 Supplementary Front Lens

TABLE I SPECIFICITY OF UNRESPONSIVE STATE ESTABLISHED BY FEEDINGS

Substance Fed	Animals per Group	Subsequent Sensitizing Procedures	Final Contact Tests	
			DNCB	o CBC
2 4 dinitrochlorobenzene (DNCB)	10	DNCB and *o* CBC	prac 0 to +±	+++++±
ortho chlorobenzoyl chloride (*o* CBC)	8	DNCB and *o* CBC	+++++	+ to +±
None*	8	DNCB and *o* CBC	++++	+++++±

* Feedings of corn oil or olive oil are indifferent

Legend + even faint pinkishness +± between faint and pale pink ++++ pink and slightly elevated +++++ bright pink well elevated prac 0 practically zero

that animals fed dinitrochlorobenzene resisted an active course of sensitization with it but not with orthochlorobenzoyl chloride and vice versa on the other hand animals that had been fed neither of these allergens but had undergone the double sensitization became sensitized to both equally

Picryl chloride was chosen over dinitrochlorobenzene in order to study antibody formation since the former compound is destined to produce circulating antibody commencing some days after onset of contact type dermal hypersensitivity The first experiment of this type was quite simple but rigorous animals receiving 15 feedings of picryl chloride (and others fed with olive oil) were given 12 intracutaneous injections of picryl chloride rested for weeks and finally were injected intravenously with the picrylated proteins of whole guinea pig sera The result was surprising The intracutaneous injections of picryl chloride (30 µg given during the course of days) sufficed to render control animals definitely anaphylactic when tested with 5 mg or 2 mg doses In contrast the fed animals showed at best *possible traces* even when 10 mg of the same material was injected The fed animals had handled the picryl chloride that had been introduced into their skins in such wise that they did not become anaphylactically sensitive A more searching experiment was undertaken next Animals were fed then (along with controls) injected with 5 mg of picrylated guinea pig serum proteins intraperitoneally on 5 occasions After a rest period of 6 days another type of picrylated protein (picryl casein) was injected intravenously to utilize anaphylaxis in determining the presence or absence of specific antipicryl antibodies Picrylated guinea pig serum acted quite adequately as antigen in normal animals leading to anaphylactic type hypersensitivity doses of 5 mg down to 1 mg of picrylated casein were fatal But, as before animals that

had been fed showed only traces of anaphylactic reactivity, even though the challenge dose was increased

The alteration with respect to the development of contact type hypersensitivity that is induced by feeding chemical allergens is profound An excellent illustration is provided by experiments in which a special method worked out years ago in our laboratory to supersensitize guinea pigs was employed[8] The method of sensitization called the combination method consists in a primary stimulation secured by the injection in a water in oil emulsion of picrylated guinea pig erythrocyte stromata having tubercle bacilli suspended in the outer oily phase[15] Following this primary stimulus the simple chemical itself is applied to the skin at certain intervals with the surprising consequence that the degree of dermal hypersensitivity increases many fold Guinea pigs commonly become sensitive to contact with 1 15 000 of picryl chloride in olive oil and some show a definite degree of reactivity to 1 45 000 Applied to animals that have had feedings of picryl chloride this intensive allergizing stimulus does indeed effect a break in the resistance that these animals have acquired The degree of sensitivity that is imposed on animals by this method is however far less than is imposed on normal animals by means of a few injections of 5 μg of picryl chloride The discrepancy between the sensitivities possessed by control versus fed animals was fifty to a hundred fold that is fed animals came to react to contact with 1 300 controls to contact with 1 15 000 to 1 45 000 of picryl chloride in olive oil[3] Quite evidently therefore the process of feeding makes a qualitative change in the resistance of the animals to sensitization

It turned out to be much more easy to 'upset the inability of a fed animal to produce ordinary circulating antibody possessing the specificity of the fed allergen One had only to inject a strongly antigenic protein to which picryl groupings had been coupled in order to induce the production of antibodies and anaphylactic sensitization This was accomplished readily by injecting picrylated bovine gamma globulin adsorbed on aluminum hydroxide[3] In fact fed animals so treated came to produce antibody perhaps 48 hours before their normal brothers did in grouped experiments Taken at the proper time the serum of either fed or normal animals would show precipitins in capillary tubes at concentrations (absolute) of picrylated casein of 1 500 down to 1 10 000 This result stands in contrast to those that we mentioned previously, in which definite resistance is found to formation of circulating antibodies that are directed against picrylated derivates when attempts are made to invoke anaphylactic sensitivity either by means of protein carriers that possess limited antigenicity (such as altered homologous proteins) or by a series of injections of picryl chloride into the skin[7] It is found however that even though the fed animals can be forced into producing antipicryl anti

body by a strong antigen such as picrylated bov...
animals remain resistant to development of contac...
They do not acquire such a state automatically...
antipicryl bovine gamma globulin antibody nor...
cutaneous injections of picryl chloride laid dow...
of antibody give rise to contact type hypersensi...

Experiments were designed with a view to c... hension of the reasons for the immunologic unres... from fed animals failed to exhibit any blocking... sensitin into animals undergoing active sensitizat... development of dermal sensitization[9, 10] Other c... at the question of the resistance of these animals t... tions of passive cutaneous anaphylaxis and (*b*) to... sensitivity imposed by means of white cells h... sensitized normal animals It was determined that... no different from normal animals in retaining in... dose of antibody prepared to 4 dinitrochlorob... react with a typical 3 to 8 minute maximal react... protein was injected intravenously on the follo... found that white blood cells or lymph node cel... injected into fed and well resistant animals v... mals dermally hypersensitive just as efficiently as... condition upon normal animals * The conclu... inhibited animals do not possess a mechanism that... that might acquire the specific potentiality of... hypersensitivity One difference there was... type hypersensitivity is imposed by means of v... often show a biphasic type of response involv... later more permanent phase of hypersensitivity...

It must be pointed out that animal receiving ... picryl antibody will show transient reactions on the ... made with olive oil solutions of the allergen Unl... reactions can be differentiated from those of the acti...
Also tending to differentiate between a capacity f... development of contact type hypersensitivity are the ... labile sensitizers such as phthalic anhydride when sen... of a series of intracutaneous injections of the chem... intracutaneous injection of chemicals of such high ... speedily with protein — suffices to form conjugates ... to observe the acquisition of Arthus type reactivity ... tions it is seen that the reactions in the contr... more) pendulous with edema and eccentric while ... similar in type are small The delayed type percu... differentiate rather sharply between fed and ... feedings have resulted in a state of partial inhibitio... interfering with the inhibition of delayed type hyper... tion is required

thirteenth day and may continue for several months[9] In contrast animals having the characteristics of immunologic unresponsiveness exhibit a typical first response (during 1 to 6 days) but do not appear to show the second more permanent phase unless large volumes of cells are injected Our observation probably would call for some modification in the concept of the transfer factor that is postulated by Pappenheimer and Lawrence[16, 18] to pass from one cell generation to daughter cells and so perpetuate hypersensitivity

The next question to be answered was this, although conceptually it is not far from the point just raised Would white cells removed from the milieu of the immunologically unresponsive guinea pigs be able to sensitize normal animals The answer is decidedly in the negative[2] Working at extremely low donor recipient ratio for sensitized *normal* donors (1 1 or 1 5 1) it turned out that dermal hypersensitivity was readily transferred to picryl chloride and under conditions in which a concomitant transfer of tuberculin reactivity occurred but only at the threshold level With cells removed from fed donors that had not responded well to the combination method of sensitization even a donor ratio of 3 1 or 4 1 imposed no dermal reactivity to picryl chloride despite the fact that reactivity to tuberculin (for which the animal was not immunologically unresponsive) actually arose Apart from the somewhat accelerated response of animals fed picryl chloride to stimulation by picrylated bovine gamma globulin one would conclude that the cells of fed and challenged animals had never received a directed immunological stimulus a situation which would be consonant with the concept of immunologic unresponsiveness Nor have we found evidence that the cells of resistant animals confer resistance upon normal animals

We have already mentioned that so far as the use of C^{14} labeled chemical allergens has gone in the hands of Dr Ritts there is exceedingly little evidence for the retention of large amounts of picryl groupings Autoradiographs are encountered with extreme difficulty and only after many months of contact with stripping film Consonant with this were experiments made to ascertain the existence of antigenic sites within our immunologically unresponsive animals that would be capable of reacting with anti picryl antibody[2] Stark for example has inferred that the continued presence of pneumococcal polysaccharide in mice may indeed render them able to take up specific antibody, he would find the conclusion logical that the animals are not actually immunologically unresponsive but produce antibody slowly that is removed by the antigenic depots[1, 20] For our study anti picryl antibody prepared in guinea pigs by two different methods was employed Guinea pig antisera were injected into animals weighing between 450 and 850 Gm in carefully paired groups,

with the intention to supply such a small amount of antibody that loss by adsorption onto tissues would not be overlooked One animal of each pair was a fed animal the other was a normal counterpart After the intravenous injection, blood samples were removed by bleedings from the ear or by cardiac puncture at selected time intervals in such wise that the well being of the animal was not disturbed The quantity of antibody injected was designed to establish such an initial concentration that the serum taken from a normal recipient at 15 minutes could be diluted 1 6 and give on the average reactions of 14 mm diameter when tested by passive cutaneous anaphylaxis By this test it was found that immunologically resistant animals did not take up antibody from the blood stream at a faster rate either initially or over the course of 4 to 6 days in any way different from the equilibration of antibody observed in the paired normal animal (There are technical difficulties in assessing the amount of antibody in any sample bleeding by passive cutaneous anaphylaxis which are not to be resolved lightly In the case of the procedure of passive cutaneous anaphylaxis the assessment of relative antibody concentrations in different samples of serum goes along well enough provided that one can dilute a sample in saline 1 8 or more When on the contrary it becomes necessary to use serum diluted 1 3 or 1 or undiluted difficulties arise some samples of serum 'mask' their antibody content and this ability varies from the serum of one animal to another in a way that we are not yet able to predict But when equivalent dilutions were laid down at 1 3 or 1 5 or 1 8 depending upon the initial charge of antibody no consistent differences could be determined between the response of fed and normal animals in studies of many pairs of animals)

In alternate tests we examined the bleedings by Boyden s tanned cell agglutination reaction using sheep cells that were coated with picrylated protein prepared from crystallized bovine serum albumin In this test also there is some degree of irregularity when one uses sera in which the antibody concentration permits only slight dilution It is true also that the end points vary from one batch of coated cells to another and consequently the titers secured depend on the particular test Despite these working conditions it was quite apparent that antibody circulated in both resistant and normal animals in close to the same concentration even at the end of 4 or 6 days For example at 216 hours the final agglutinating titer of the antibody circulating in a control animal might be 1 40 and in a fed animal between 1 o and 1 40 or it might be 1 10 in a control animal and clearly 1 20 in a fed animal

At this point we should like to proffer a question What if we *had* found that we could adsorb antibody in the tissues of fed animals? It is likely that we could have arrived at an hypothesis parallel to Stark s that antigen (pneumococcal polysaccharide) injected into mice may not

paralyze them but leave them able to produce and absorb antipneumococcal antibody In our own case where significant antigenic depots are not found to occur, we have no support for concluding that animals actually respond to an allergenic stimulus but unbekownst conceal all evidence of immunological fulfillment Could we not therefore allege that the immunologic unresponsiveness of the mouse may rest not upon the bulk of pneumococcal polysaccharide held in its tissues but rather on the same basis that appears to operate in determining the unresponsiveness of our guinea pigs?

Our general conclusions are given at the start of this paper We are intrigued with the idea that animals can be prepared not only to show a quite high degree of immunologic unresponsiveness' but also evidently, to exhibit some more or less stabilized intermediate position with respect to their ability to respond to the induction of dermal hypersensitivity Several attempts have been made to transpose our techniques to human beings with the intent of rendering them resistant to experimental sensitization with simple chemicals The fact that the procedures utilized so far have failed — application of a few subsensitizing applications of dinitrochlorobenzene to the skin[23] the feeding of krameria extract[13] the feeding of dinitrochlorobenzene and poison ivy extract[14] — can hardly set aside the biological principle involved While dosage toxicity, previous exposure body weight proper route of administration and timing are some of the factors to be considered if one would try to construct a practical procedure the real goal is to acquire an understanding of the reasons for effecting a shunt in the immunological apparatus We would return to the idea that our experiences which have been secured with the use of adult animals may not be far removed fundamentally from the kind of unresponsiveness that Medawar and his group have secured in animals by injecting them in late fetal life

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*Further Observations on Immunologic Unresponsiveness Induced by Type I Pneumococcal Polysaccharide**

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The problem of 'immunological paralysis' first stated by Felton and Ottinger* in 194 , has subsequently been investigated in a variety of ways by different research workers. With the development of the present understanding of the phenomenon certain reassessments are necessary. They may be summarized as follows:

(1) The term immunological paralysis is misleading and the phenomenon might better be referred to as immunological masking since the failure to demonstrate an antibody response does not imply its absence.

( ) The lack of the immune response to large dosages of pneumococcal polysaccharide is the result of neutralization of antibodies as fast as they are formed rather than of any interference with antibody forming mechanisms.

(3) The phenomenon once considered to be a peculiar characteristic of the polysaccharides of the pneumococcus is capable of being elicited by other antigenic materials under certain experimental conditions though the mechanisms involved may be different in some cases.

(4) Whenever an antigen persists in the animal for a greater or less period after parenteral introduction whether due to the size of dosage used or to the nature of the antigen probable interference with the immune response is likely to follow.

(5) The antigens used to produce the phenomenon undergo changes with the passage of time either from elimination by host metabolism or from reaction with antibody or both. These changes result either in a

* This investigation was supported in part by Research Grant [illegible] from the Division of Research Grants of the National Institutes of Health, U.S. Public Health Service. Appreciation is also expressed to Miami University for granting time for the work and to the Department of Bacteriology, University of Michigan, for provision of space to carry on the project.

reduction in the residual amount of the particular antigen used or in reduction of its antigenicity

(6) Persistence of antigen after parenteral introduction a characteristic of pneumococcal polysaccharides is being demonstrated with other types of antigenic material though to a smaller degree

Brieger and Ehrlich[1] called attention to the decrease in circulating antibody which follows introduction of antigen in immunized animals in 1893 Hektoen and Welker[8] confirmed the drop in antiserum titer and the specificity of the reaction by showing that the introduction of hemoglobin or ovalbumin antigens in immunized animals caused the temporary disappearance of homologous precipitin reactions previously present Titers were not changed by heterologous antigens

Downie[4] observed that type I pneumococcal polysaccharide (SI) in dosages of 0 0 5 μg gave good protection to mice challenged with the homologous culture but that 100 μg of SI gave no protection at the end of 1 week though some protection was present at the end of 3 weeks

Considerable evidence has accumulated to indicate that failure to demonstrate tissue or circulating antibody is often the result of neutralization by reaction with residual antigen rather than of any interference with antibody formation Hektoen and Welker s results point to this interpretation Kaplan Coons and Deane[9] in a study of the cellular distribution of SII and SIII state Newly formed antibody is continuously neutralized as a result of the persistence of antigen both in the tissues and in the circulating blood Uyeda[15] found that mice paralyzed by SI respond normally to immunizing dosages of SIII and SVIII and that mice paralyzed by SIII and SVIII respond normally to SI However animals paralyzed by minimal dosages of SIII cannot be immunized by SVIII but animals paralyzed by minimal dosages of SVIII could be immunized by SIII He interpreted the results obtained with SIII and SVIII to be the result of close chemical relationship between the two Both SIII and SVIII contain aldobionic acid SIII exclusively and SVIII containing glucose in the ratio of two glucose molecules to one of aldobionic acid He considered the aldobionic acid to be the reactive group and interpreted the failure of minimal dosages of SVIII to paralyze against SIII to be due to the smaller amount of aldobionic acid present Morgan Watson and Cromartie[13] demonstrated that immunological paralysis induced by SII did not prevent a normal response to SVI

Felton[5] by use of precipitin tests demonstrated that SI remains in the tissues of the mouse for at least 52 weeks The dosage level was 500 μg It was noted that a decrease in antigenicity of recovered SI occurred during the course of the experiment Stark[14] prepared SI labeled with C^{14} and found no significant decrease in radioactivity of splenic extracts

of SI a year after injection of 500 μg The antigenicity of the recovered SI was however, markedly reduced Dixon, Maurer and Weigle [8] demonstrated that tissue fixed SIII was capable of uniting with I^{131} labeled ar 1 SIII rabbit serum gamma globulin and that it was promptly metabolized off leaving the SIII free to unite with antibodies as fast as they were formed

Ørskov [21] has demonstrated unresponsiveness to the capsular material of *Klebsiella pneumoniae* when used in excessive dosages Dixon and Maurer [2] found that unresponsiveness to heterologous proteins persists as long as the antigen can be detected but not longer This would be anticipated if lack of response is caused by degradation of antibodies by reaction with the antigen Garvey and Campbell [7] using S^{35} labeled hemocyanin and S^{35} labeled bovine serum albumin as antigens have shown that small amounts of the antigen persist for 4 to 10 months and from the rate of loss at the close of the experiments might be expected to persist for several years Perhaps the masking of an immune response by the polysaccharides of the pneumococcus is unusual only in the duration and the order of magnitude of their persistence in animal tissues

Our recent experimental work has been designed to elucidate the mechanism involved in the decrease of antigenicity observed in SI recovered from mice receiving larger dosages than required for immunization

We have adopted as a working hypothesis that some change which is as yet unidentified takes place in SI as a result of antigen antibody reaction If this be true it follows that the antigen change should bear a definite relationship to the amount of antibody present Two approaches have been used to test this hypothesis The first is based on inhibition of antibody formation which should decrease the rate of antigenicity loss The second approach involves the use of antiserum to increase the concentration of antibody which should lead to an increased rate of loss of antigenicity

It is well established that whole body x irradiation in adequate dosages suppresses antibody production for a period of time One hundred mice averaging 5 Gm in weight were exposed to whole body x irradiation using a dosage of 4 5 r The lethality of this amount of irradiation in this experiment approximated 7 per cent at the end of 30 days Twenty four hours after irradiation the irradiated mice and an equal number of normal mice were injected intraperitoneally with 100 μg of highly purified SI in 1 0 ml of saline Twenty four hours after injection of SI 9 mice were sacrificed and the spleens were removed and weighed After addition of 5 0 ml of water per gram of spleen grinding was accomplished by use of a Ten Broeck tissue grinder After grinding extraction of SI was made at 4 to 5 C for 3 hours, following which the solids were centrifuged out at 1300 RCF Several appropriate twofold dilutions of the supernatant

were made and injected into mice for antigenic analysis by the method of Sandage and Stark[13] From these data 50 per cent protective doses were calculated by the method of Reed and Muench[12] Comparable analyses were made after 10 o and 30 days The experiment was terminated at the end of 30 days as inhibition of antibody formation by x irradiation is relatively transient

It was previously established that 0 00255 μg of this lot of SI is required to protect 50 per cent of mice challenged by 1 0 ml of 10^{-7} dilution of a 16 hour culture of type I pneumococcus injected intraperitoneally in tryptose phosphate broth This represents a challenge dose of approxi mately 100 MLD The micrograms of SI present were calculated per spleen in order that the error introduced by the marked decrease in weight followed by gradual recovery which results from x irradiation might be minimized It will be noted in Figure 1 that x irradiated animals

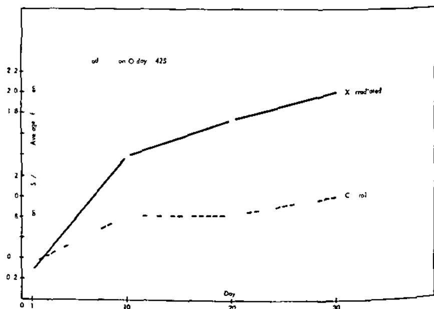


FIGURE 1 The effect of x irradiation of mice on the antigenicity of SI recovered in splenic extract

show a consistently larger amount of *antigenic* SI per spleen than do normal animals This would be expected if the antigen antibody reaction brings about reduction in the antigenicity of SI recovered from the animal We interpret the observed increase in antigenicity with time probably to be due to the time required for equilibration of SI in the various tissues of the mouse

The use of x irradiation to inhibit antibody formation in response to SI has the serious defect of being transient in nature In a previous study we found that the greatest decrease in antigenicity of SI recovered from the spleens of animals took place after approximately 2 months hence the results reported here have limited value due to the relatively brief duration of inhibition of antibody synthesis following x irradiation

Dixon Maurer and Weigle [8] demonstrated that rabbit anti SIII gamma globulin disappears from mice which have been given 0 5 mg of SIII more rapidly than from normal mice This would be consistent with the hypothesis that the lack of immunological response which follows the introduction of a masking dosage of the antigen may be the result of removal of antibody as fast as formed by reaction with surplus antigen If decreased antigenicity of SI recovered from mice is due to such a reaction the antigenicity decrease should be more rapid in mice treated with rabbit antiserum following introduction of the masking dosage of SI

We therefore obtained an unfractionated rabbit anti SI serum * which gave passive mouse protection in dosages of 1/1000 ml to all animals challenged with 100 MLD Its agglutination titer was 1 160 Antibody nitrogen determination showed 640 μg /ml

One hundred fifty mice were injected intraperitoneally with 100 μg of SI in 1 0 ml of saline using the same lot of SI as in the previous experiment (PD_{50} = 0 00155 μg) Fifteen days later the spleens were removed from 10 mice 5 0 ml of water per gram of spleen was added and they were ground in a Ten Broeck tissue grinder Extraction and preparation of dilutions were carried out as with the x irradiated series previously described A 10 0 sample was removed before centrifugation for determination of the dry weight of solids present in order that the total dry weight of the ten spleens might be calculated

The 50 per cent protective dilution of splenic extract was determined by injection of 10 mice in each group with a suitable dilution using five or six different twofold dilutions and calculating the end point by the method of Reed and Muench The antigenic SI present in splenic extract the amount needed to give 50 per cent protection (0 00155 μg) the dilution factor protecting 50 per cent of the animals challenged and the dry weight of spleens extracted were used in calculation of the antigenic SI per gram dry weight of spleen Results are expressed in micrograms of antigenic SI per gram dry weight of spleen

The remaining mice were then separated into two groups one to be treated with antiserum the other as an untreated control group The antiserum group was injected on the same day as the first assay using antiserum in a dosage giving 64 μg of antibody nitrogen (Ab N) Three

* Dr Arthur Johnson kindly supplied this material and the data concerning it

injections of this dosage were made at 7 day intervals On the thirty second day following the injection of 100 μg of SI and 3 days after the last injection of antiserum, 10 mice of each group were sacrificed and the antigenicity of the residual SI was determined as before The Ab-N injected totaled 19 2 μg at this time

The 3 day interval between the last injection of antiserum and determination of the residual antigenicity of SI present in splenic extract allowed time for reduction of circulating antibody to a marked degree Dixon Maurer and Weigle[8] have demonstrated that metabolism of antibody from antigen antibody complex is rapid, only about 30 per cent of that introduced passively remained at the end of 3 days Furthermore the dilutions of extract necessary to determine the 50 per cent end point were sufficiently great to obviate any effect of passive transfer of immunity to the mice used for assay

At this time the antiserum injections were increased to 12 8 μg of antibody nitrogen at each injection Injections were continued at 7 day intervals and on the fifty third day 10 mice from each group were sacrificed and the amount of antigenic SI was determined as before The Ab N injected to this time totaled 51 μg

Antiserum injections of 1 8 μg of Ab N were continued at 7 day intervals until the seventieth day The Ab N injected totaled 89 6 μg at this time On the seventy third day 10 mice from each group were sacrificed and the antigenicity of the residual SI was determined

The results obtained are shown in Figure . It will be noted that the amount of antigenic SI present in the spleen increased, probably due to equilibration in the tissues of the mouse until the fiftieth day in the control group after which a decrease became evident In contrast to this mice which had received 19 . μg of Ab N show only a slight increase at the thirty-second day after which the larger dosage of Ab N used led to a more rapid decrease which reached a very low level by the seventy third day This is in agreement with our previous work which showed that the rate of decrease in antigenicity as determined by the 50 per cent protective dosage end point was greatly accelerated after about months

It will be noted that the decrease in antigenicity in the control group appears only after the fifty third day and then is slight In contrast mice receiving antiserum injections began to show the decrease earlier and to a much greater degree It will also be noted that doubling the antiserum dosage is accompanied by a marked decrease in antigenicity of SI splenic extract as compared with the smaller dosage This later decrease in the antiserum group is complicated by the passage of additional time during which the effect of antibody formation by the mouse is a factor However in comparison with the control it is evident that the injection of Ab N accelerated decrease in antigenicity of the SI originally injected

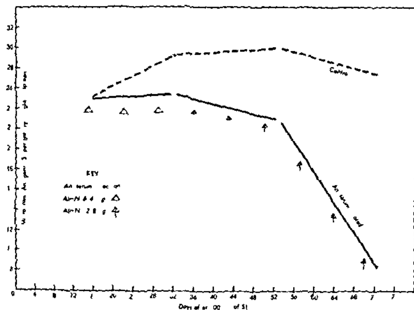


FIGURE 2 The effect of antiserum treatment of mice on the antigenicity of SI recovered in splenic extract

To establish some concept of the rate of antigen antibody combination *in vivo* the following experiment was performed A group of mice was immunized by intraperitoneal injection of 0 5 μg of SI Previous tests have shown that this dosage gives a high degree of immunity by the fifth day

Five days after immunization the mice were divided into five groups One group was retained as a control group and received no additional injections of SI The other four groups were given 15 μg 30 μg 60 μg and 120 μg intraperitoneally One hour after these injections 10 mice from each group were challenged by intraperitoneal injection of 100 MLD of the homologous culture Other groups were challenged after 6 hours 24 hours 2 days 5 days, and 50 days following injection of the various dosages of SI Results are given in Table I

It is evident from these results that previously formed antibody is quickly neutralized even by dosages far below those normally considered to be paralytic (the negative phase of Hektoen and Welker) but that this is very transient with small dosages of SI As the dosage is increased the protective antibody neutralization endures for a longer time as would be expected if an antigen antibody reaction occurs *in vivo* and results in removal of free antibody No dosage used represents a full paralyzing dosage and it might be expected that resistance to infection would ulti

TABLE 1 THE EFFECT OF ADDITIONAL SI INJECTION ON THE RESISTANCE OF PREVIOUSLY IMMUNIZED MICE

Challenge Time	SI Dosage 5 Days After Immunization					
	0 μg	15 μg	30 μg	60 μg	120 μg	Culture Controls
1 hour	10/10*	0/10	0/10	1/9	0/10	0/5
6 hours	10/10	6/10	3/10	2/10	0/10	0/5
1 day	10/10	7/10	3/10	1/10	0/10	0/5
2 days	8/10	5/10	2/9	1/10	0/10	0/5
5 days	9/10	6/10	7/10	2/10	3/9	0/5
50 days	8/10	5/10	4/10	3/10	2/9	0/5

Survivors 96 hours after challenge / number challenged

mately reappear to increasing degree even with a dosage of 120 μg The reappearance of some protection is very rapid following its disappearance in the group given 15 μg

The rapidity of the absorption and distribution of SI in animal tissue is evidenced by the lack of resistance to infection which occurs 1 hour after introduction of as little as 15 μg of SI in immunized mice It must be remembered that the resulting infection following challenge does require approximately 48 hours to become serious enough to kill most of the animals challenged This of course allows additional time for neutralization of antibody However if any significant effective antibody were present early in the infective process, when the number of organisms is small one might expect the infection to fail to develop

All of these experiments were designed to further clarify mechanisms involved in antigen antibody reactions displayed in response to SI It should be emphasized that they support, but do not prove the hypothesis that *in vivo* antigen antibody reactions are probably responsible for changes in the antigen as well as removal of antibody What the antigenic change may be must wait for more exacting chemical and stereochemical studies

In many respects the behavior of SI when introduced in amounts which mask the immune response shows many of the characteristics of an enzyme substrate type of reaction In such an analogy, the SI would be comparable to the enzyme in that its disappearance as an antigen is extremely slow in taking place just as an enzyme may repeat its activity time after time but eventually decays and loses its catalytic effect The antibody would be comparable to the substrate as the same SI may remove antibody time after time resulting in the absence of any protective effect Even the mechanisms involved show striking similarity The enzyme unites with the substrate leads to its degradation is freed and unites with other molecules of substrate This sequence is repeated again and

again with the result that the substrate is constantly disappearing just as free antibody disappears in the presence of surplus antigen The enzyme or the SI is at the same time though much more slowly being degraded to an inactive state

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*Immunologic Unresponsiveness to Protein Antigens**

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Immunologic unresponsiveness to serum protein antigens has been observed under a variety of conditions It can be found as a part of the nonspecific immunologic unresponsiveness seen in neonatal rabbits[7] and in rabbits recently subjected to whole body x radiation[6] In addition it can be specifically induced by the administration of protein antigens in small amounts to neonatal rabbits[1 2 13 14 17] and in larger amounts to normal or x radiated adult rabbits[3] While the nonspecific unresponsiveness of neonatal and x radiated adult rabbits is temporary the duration of the antigen induced unresponsiveness may be temporary or permanent depending on the conditions under which the experiment is performed[3] Although the failure of the rabbits to produce a detectable immune response is characteristic of all these types of unresponsiveness the nature of these types appears to differ It is the intent of this paper to describe the various types of antigen induced unresponsiveness and to compare them with the nonspecific unresponsiveness of neonatal and x radiated rabbits

Antigen induced Unresponsiveness Initiated in Neonatal Rabbits

This type of unresponsiveness is observed in mature rabbits which are injected with the antigen shortly after birth Since rabbits possessing this type of unresponsiveness have been called tolerant rabbits[2] this term will also be used in the present paper to differentiate rabbits possessing unresponsiveness initiated by neonatal injections of the antigen from rabbits possessing other types of unresponsiveness In early experiments in our laboratory a total of 40 to 50 Gm of foreign serum proteins were injected into the rabbits over a period of approximately 100 days begin

* This study which is Publication No 155 of the Department of Pathology University of Pittsburgh School of Medicine Pittsburgh was supported by U S Atomic Energy Contract AT(30 1) 1205 Dr Weigle is a Public Health Service Research Fellow of the National Institute of Neurological Diseases and Blindness

ning on the first day after birth[3] Five rabbits received whole human plasma and 3 rabbits received bovine serum albumin (BSA) The rabbits which were injected with human plasma did not give an immune response to either human serum albumin (HSA) or human gamma globulin (HGG) when last tested 6½ months after the injections were discontinued Likewise, the rabbits which were injected with BSA gave no immune response to BSA during the term of the experiment which ended 5 months after the injections of the antigen were stopped The unresponsiveness observed during these periods was specific since the rabbits did make an immune response to other protein antigens

Unresponsiveness was also observed in rabbits which received much smaller injections of heterologous proteins during a very short period following birth These rabbits were injected with a total of 500 mg of bovine gamma globulin (BGG) or 500 mg of both BGG and BSA or 500 mg of HSA during the first 5 days after birth Twenty four out of 28 rabbits injected shortly after birth with BGG did not respond to BGG when tested 3 months later Sixteen out of 16 rabbits injected with BSA and 3 out of 3 injected with HSA did not make an immune response to the respective antigens when tested 3 months later All of the animals were still unresponsive when last tested 3 to 6 months after birth These rabbits which were injected shortly after birth with 500 mg of BSA were injected 6 months later with 25 mg of BSA in adjuvant containing tubercle bacilli When these rabbits were tested to 3 weeks later they possessed neither circulating anti BGG nor delayed skin sensitivity to BGG The rapid immune elimination of I^{131} labeled protein antigens was used in all of the studies as the indicator of an immune response [2]

Whether the unresponsiveness initiated by a previous contact with protein antigens in neonatal life is the result of the persistence of the antigen has always been a question Smith has found that the duration of unresponsiveness in rabbits injected with antigen during the neonatal period is related both to the amount of antigen initially injected and also to subsequent injections of the antigen after the neonatal period[18] This lends support to the thesis that the unresponsiveness is dependent on the persistence of the antigen On the other hand there are available data which indicate that persistence of antigen may not be essential First protein tolerant rabbits do not eliminate passively administered antibody from their blood, any more rapidly than do normal rabbits[3] indicating the absence of a detectable combination of transferred antibody with persisting antigen Second if a trace of protein antigen does persist in cells of the tolerant rabbits it does not inhibit normal or sensitized lymph node cells from making an immune response when transferred to the tolerant rabbit[26] Third if there is any protein antigen which

persists in the "tolerant" rabbit it is not detectable by available methods If one accepts the 4 day half life for the rate of elimination of BSA or HSA from the circulation[22] as being representative of the elimination of these antigens from the entire rabbit the maximum amount of antigen present in rabbits made "tolerant" by injections totaling 500 mg during the first 5 days of life would be less than 5×10^{-6} μg 6 months after the injection For BGG less than 1 molecule would remain after 6 months since the half life of its rate of elimination in rabbits is only 2 2 to 2 3 days[22] In absence of evidence for the persistence of detectable antigen in "tolerant" rabbits it may be that this type of unresponsiveness is not the result of the persistence of the antigen

It is generally assumed that the persistence of the antigen in mice injected with large amounts of pneumococcal polysaccharide is responsible for the "immune paralysis" which results Large amounts of the polysaccharide have been shown to persist in the mice for long periods of time[12, 19] That this persisting polysaccharide is capable of removing passively administered antibody from the circulation has also been shown[4] With the polysaccharide mouse system it appears that the failure to detect an immune response could result from the neutralization of the antibody by the persisting antigen[4, 18, 19] However it is obvious that this system differs in a number of ways from the unresponsiveness which is initiated in neonatal rabbits by protein

Protein "tolerant" rabbits may recognize the protein to which they are unresponsive as being nonantigenic in a similar manner as they do to their own proteins as has been previously suggested[3] However the "tolerant" rabbit does recognize the protein as being foreign on a non immunologic basis[23] This is made clear by the fact that the half lives of the rate of exponential elimination (the slow elimination following equilibration between intra and extra vascular fluids and prior to rapid immune elimination) of BSA HSA and BGG from normal or "tolerant" rabbits were shorter than the half lives of either the homologous albumin (RSA) or gamma globulin (RGG) (Table I) This can best be seen with BGG which has a half life of 2 2 to 2 3 days in either normal or "tolerant" rabbits whereas the half life of the homologous globulin is 5 0 days Also the half life of HSA and BSA in normal and "tolerant" rabbits is 3 9 to 4 3 days whereas the half life of the homologous albumin is 5 7 days

The value reported by Cinader and Dubert for the half life of HSA in "tolerant" rabbits is in disagreement with that given in Table I Their half life for HSA during the exponential phase was the same as that found for the homologous albumin which was longer than that observed for HSA in normal rabbits However these authors suggested that their observation may have been the result of genetic or nutritional differences

TABLE I HALF LIVES OF HOMOLOGOUS AND HETEROLOGOUS SERUM PROTEINS IN NORMAL, X RADIATED AND TOLERANT RABBITS

Rabbit	Protein	Interval of Measurement in Days after Injection	Average Half life in Days
Normal or x radiated	RSA		5.7
Normal	BSA	3–6	4.3 ± 0.3
BSA tolerant †	BSA	3–20	4.3 ± 0.2
Normal	HSA	3–7	4.1 ± 0.4
X radiated ‡	HSA	3–9	3.9 ± 0.3
HSA tolerant †	HSA	3–17	4.0 ± 0.4
Normal or x radiated	RGG		5.0
Normal	BGG	2–5	2.1 ± 0.2
X radiated ‡	BGG	3–9	2.2 ± 0.2
BGG tolerant †	BGG	3–16	2.3 ± 0.[illegible]

Standard deviation

† Tolerance induced by injection of 500 mg of specific antigen during first 5 days of life

‡ Received 400 r whole body x radiation [illegible] days prior to the injection of the proteins

in the two groups of rabbits Also we have found that BSA HSA and BGG have the same half life in tolerant x radiated and normal rabbits (Table I) and as mentioned above these half-lives are shorter than those of the homologous proteins Therefore it must be concluded that immune mechanisms play no role in the exponential elimination of heterologous proteins This is in agreement with other observations that the exponential rates of elimination of heterologous serum proteins are not related to their degrees of antigenicity [8, 3, 4]

ANTIGEN INDUCED UNRESPONSIVENESS INITIATED IN NORMAL ADULT RABBITS

This type of unresponsiveness is obtained by injecting rabbits with large amounts of heterologous serum proteins over a long period of time[3] Rabbits treated in this manner failed to make an immune response to the foreign proteins until after the disappearance of the protein from the circulation One group of normal adult rabbits was injected over a 53-day period with a total of 23 Gm of human plasma protein Another group was injected for a period of 43 days with a total of 18 Gm of BSA The amount of protein injected was so chosen as to approximate the amount of corresponding autologous protein synthesized by the host during these periods Rabbits injected with human plasma developed neither circulating anti HGG nor circulating anti HSA until [illegible] to 6 months after the injec

tions were discontinued However severe lesions of serum sickness developed within 1 to weeks after the injections began Since no antibody response to either HGG or HSA was detected in these rabbits 3 to 7 months later it is likely that the lesions were related to an antibody response to other perhaps lesser constituents of human plasma The rabbits injected with BSA did not recover their ability to make an immune response to BSA until 1 to 3 months after the injections were discontinued One out of the 5 rabbits injected with BSA was still unable to make a response to BSA one year after the injections were stopped Rabbits were also injected daily with BSA and at various intervals were sacrificed and examined for lesions of serum sickness No lesions were found in rabbits sacrificed at the end of a week course of daily injections of 3 Gm of BSA nor at the end of a 3 week course nor weeks after the termination of a 3 week course

There was no rapid immune elimination of HSA or HGG in the rabbits injected with human plasma or of BSA in those injected with BSA after the injections were discontinued Therefore it would appear that these animals were temporarily unable to make an antibody response On the other hand considerable amounts of antibody might have been produced during the periods of injections and removed as antigen antibody complexes without showing a noticeable decline in circulating antigen However this was not true 5 to 6 weeks after the injections were discontinued when the concentration of foreign protein dropped to 0 1 mg per milliliter of serum or less A small amount of antibody synthesized during this time would result in the immune elimination of this amount of protein The absence of an immune elimination of these small amounts of proteins plus the failure to observe lesions of serum sickness in the rabbits injected with BSA lends support to the contention that temporary unresponsiveness was obtained in normal rabbits infused with large amounts of foreign protein

There are certain similarities between unresponsiveness initiated by infusion of normal rabbits with heterologous serum proteins and the immune paralysis observed in adult mice injected with polysaccharide (1) Both require an injection of a relatively large amount of the antigen (2) The failure of the appearance of circulating antibody can be correlated to the persistence of antigen (3) Both can be initiated in adult animals However there is one possible difference which may be used to differentiate between these two types Immune paralysis may not be a true unresponsiveness but merely a failure to detect the response because of a neutralization of the antibody by persisting antigen whereas unresponsiveness initiated by the infusion of adult rabbits with proteins appears to be the result of the inability of the rabbit to produce antibody for a few months after cessation of injections

Antigen induced Unresponsiveness Initiated in X radiated* Adult Rabbits

This type of unresponsiveness differs from the last type in that it is produced by the infusion of x radiated adult rabbits with heterologous serum proteins rather than by the infusion of normal adult rabbits[2] Also this type of unresponsiveness is more permanent than that produced by the infusion of normal rabbits One group of x radiated adult rabbits was injected with a total of 25 Gm of human plasma protein over an 8 week period while another group was injected with a total of 21 Gm of BSA over a 7 week period Three months after the injections were discontinued the only remaining rabbit which was injected with human plasma did not make an antibody response when tested with HGG and HSA and the 3 rabbits injected with BSA did not give an immune response when tested with BSA The animal which was unresponsive to HGG and HSA did make an immune response to BSA, and likewise the 3 rabbits unresponsive to BSA gave a response when injected with BGG When last tested 6 to 9 months after the injections were stopped both of the 2 remaining rabbits (1 infused with human plasma and 1 infused with BSA) were still unresponsive to the antigens which they were infused with If one assumes in these rabbits a normal rate of catabolism of heterologous serum proteins no more than a fraction of a microgram of the proteins would be expected to be remaining at this time

While unresponsiveness induced in x radiated rabbits is similar to that induced in neonatal rabbits it differs from the unresponsiveness induced in normal adult rabbits in the following two ways (1) the unresponsiveness appears to be permanent in the x radiated rabbit whereas it is only temporary in the normal rabbit and (2) the unresponsiveness is present in the x radiated rabbit in the absence of detectable amounts of circulating antigen whereas the unresponsiveness in normal rabbits disappears when all of the antigen is catabolized

The Nature of the Nonspecific Unresponsiveness in Neonatal and X radiated Rabbits and the Specific Unresponsiveness Induced as Characterized by Cell Transfer Studies

The nonspecific unresponsiveness in neonatal and x radiated rabbits represents two different situations in addition to the antigen induced states discussed above in which there is no antibody response to soluble protein antigens (1) Neither of the nonspecific unresponsive states

* 400 r whole body x radiation was given 2 days prior to the beginning of the injections This treatment temporarily abolishes the primary antibody response to soluble proteins

requires a previous contact between the host and the antigen (2) No primary antibody response to any antigen can be elicited in these non specific states Both are of relatively short duration

While the basic metabolic defects underlying the different unresponsive states are not completely understood some insight into their nature has been afforded by cell transfer antibody production experiments In these experiments lymphoid cells capable of either primary or secondary antibody responses are transferred with the suitable antigenic stimulation to recipient rabbits The recipients either neonatal x radiated adults[5, 9, 16] or specifically unresponsive tolerant adults[26] are incapable by themselves of responding to the antigen employed The immunologic performance of the transferred cells then indicates something about the internal environment of the recipient

Neonatal Rabbits

As is well known rabbits are incapable of making an immune response during the first 2 weeks of life During this period they are also incapable of adequately supporting transferred adult lymph node cells during either a primary or a secondary response to protein antigens (Table II)[7] This deficiency is also apparent when the neonatal rabbit is supplied with adult lymph node cells sensitized with Shigella endotoxin[8] However neonatal splenic cells sensitized with *Shigella endotoxin* will produce agglutinins when transferred to x radiated adult rabbits Also lymph node cells sensitized in adult rabbits and transferred 3 days later to neonatal recipients will produce antibody[7] This would suggest either that adult sensitized lymphoid cells cannot take the necessary initial steps of an immune response in neonatal recipients or that the entire process of antibody synthesis is so slowed down in neonatal recipients that the transferred cells are rejected before a high titer of agglutinins can be achieved Consistent with the latter possibility is the observation by Sterzl that neonatal recipients appear to reject transferred cells[1] as rapidly as do x radiated adults[15] Thus it would seem that the internal environment of the neonatal rabbit is deficient for antibody production while the cells of the neonatal rabbit are capable of forming antibodies if placed in an adult environment

Sterzl[20] reported that sensitized adult lymphoid cells could produce low levels of agglutinins when transferred to neonatal rabbits The titers which we obtained with sensitized adult lymph node cells in neonatal rabbits were of approximately the same order as those reported by Sterzl but the amounts of antibody obtained with similar sensitized adult lymphocytes in x radiated adults were 10 to 20 times or more higher than those observed by us or by Sterzl in neonatal recipients Thus it would appear from the determination of agglutinin production to bacterial antigens that the neonatal rabbit while far inferior to the x radiated adult as a medium for antibody producing cells does allow the synthesis of small amounts of antibody by the transferred cells

TABLE II ANTIBODY RESPONSE BY ADULT LYMPHOCYTES TRANSFERRED TO NEONATAL X RADIATED AND SPECIFICALLY TOLERANT RABBITS

Recipient Rabbits	Primary Response		Secondary Response	
	No Cells Transferred/kg 10	Fraction of Rabbits Responding	No Cells Transferred/kg 10^5	Fraction of Rabbits Responding
Neonatal	15–20	0/18	8	0/30
X radiated *	10–25	0/14	2	103/106
Tolerant †	10–25	8/15	2	6/6

Neonatal rabbits were injected with the antigen either before or at the same time as the injection of cells

X radiated and tolerant rabbits received the antigen at the same time as the cells

* 400 r whole body x radiation 2 days prior to injection of the cells and antigen

† Tolerance induced by injection of 500 mg of specific antigen during first 5 days of life

X radiated Rabbits

Four hundred roentgen whole body x radiation given to rabbits 2 days prior to the injection of soluble protein antigens abolishes the primary antibody response but has less effect on the secondary response[6] Likewise the secondary but not the primary response to these antigens can be transferred with nonradiated adult lymph node cells to x radiated recipients (Table II) Thus the environment necessary for the secondary response is present in the x radiated rabbit whereas the environment necessary for the primary response is deficient even when the x radiated rabbit is repopulated with normal lymphoid cells

Tolerant Rabbits

Both the primary and the secondary response can be transferred with lymph node cells to tolerant rabbits[26] Eight out of 15 BGG tolerant rabbits injected with I^{131} BGG and lymphoid cells obtained from normal rabbits gave a primary immune elimination of the antigen (Table II) Also 6 out of 6 BGG or BSA tolerant rabbits injected with I^{131} antigen and lymphocytes obtained from sensitized rabbits gave an immune elimination of the antigen Therefore it is clear that all of the metabolic processes necessary for either a primary or a secondary immune response of normal cells are present in tolerant rabbits The tolerant rabbits appear to be lacking cells capable of making a specific antibody and not the environment needed to support antibody production

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*Competition of Antigens**

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Animals injected with a mixture of several antigens will often produce antibody against all the components of the mixture [21] In fact the total amount of antibody formed under such immunization conditions may greatly exceed that which is produced in response to the injection of one of the components alone [8] Nevertheless one encounters situations in which the immunogenicity of one antigen appears to be impaired by the previous simultaneous or subsequent injection of one or more other antigens Such interference with the immune response to one antigen is termed 'competition of antigens [30] Competition of antigens (CA) may manifest itself in varying degrees ranging from complete suppression to a mere delay in the attainment of the maximum level of antibody observed in controls Among the many variables that require attention in its study are the genetic constitution and physiological status of the test animals, the route and method of immunization the absolute amounts and relative proportions of the antigens injected past immunization history and the kind of antigen selected

Susceptibility to CA appears not to be associated with any well defined group of antigens or animals This is illustrated by the observations summarized in Table I, into which is condensed a substantial portion of the factual evidence on the subject of CA To this list one may add the statistical evidence which shows that Rh negative mothers are more readily sensitized by Rh positive fetuses if no other incompatibility exists between maternal and fetal blood [20]

In some instances CA has been observed in the absence of measurable antibody production against the competing antigen [1,4] In both cases cited it seems possible that antibody formation against contaminants of the competing antigen did occur but escaped detection for technical reasons In passing it should be mentioned that the concept of CA has been extended to competition between determinant groupings either natural

* This investigation was supported in part by Grant E-19 5 from the National Institute of Allergy and Infectious Diseases National Institutes of Health U.S Public Health Service

TABLE I SUMMARY OF OBSERVATIONS ON COMPETITION OF ANTIGENS

Inhibited Antigen	Inhibiting Antigen	Test Animal	Injection of Inhibiting Antigen	Reference
Serum albumin (horse beef)	Serum globulins (horse beef)	Rabbit	Simultaneous	30
Serum albumin (horse)	Serum globulins (horse)	Guinea Pig	Simultaneous	12 13
Serum albumin (beef)	Gamma globulin (human)	Chicken	Simultaneous	1
Beef serum	Horse serum	Guinea Pig	Prior	7
Gamma globulin (rabbit)	Serum globulins (rabbit)	Guinea Pig	Simultaneous	2 16
	Hemocyanin	Guinea Pig	Simultaneous*	2 16
Egg albumin	Dog serum	Rabbit	Simultaneous	27
Diphtheria toxin (toxoid)	Horse goat beef serum	Rabbit	Simultaneous	18 19
		Guinea Pig	Prior	18 19
Diphtheria toxoid	Miscellaneous other bacterial toxins	Horse	After	4
Tetanus toxoid	Diphtheria toxoid	Guinea Pig	Prior or Simultaneous*	3 6
Diphtheria toxoid *H pertussis*	Tetanus toxoid	Guinea Pig	Simultaneous	3
Tetanus toxoid	Diphtheria toxoid	Man	Simultaneous*	10
Strain specific antigens of influenza virus	Common antigens of influenza virus	Man	Simultaneous*	13
S typhosa *V cholerae*	Serum (horse pig) alcoholic or aqueous extract of guinea pig kidney	Rabbit	Simultaneous	24 29
Sheep erythrocytes	Serum (horse hog)	Rabbit	Simultaneous by same or different route	24
V cholerae	*S typhosa*	Rabbit	Simultaneous	17
Forssman antigen	Species specific lipoid of guinea pig erythrocytes	Rabbit	Simultaneous	37
Organ specific lens antigen	Forssman antigen	Rabbit	Simultaneous	38
Serum lipoids	Serum proteins	Rabbit	Simultaneous	33 35
Alcohol soluble kidney antigen	Forssman antigen	Rabbit	Simultaneous	26
Cholesterin	Lecithin	Rabbit	Simultaneous	31 32

* After previous contact with inhibiting antigen

or artificially introduced into the antigen but with one possible exception there appears to be no published account of a systematic study of this aspect of the problem

Not included in Table I are the numerous negative findings such as those pertaining to experiences with mixed bacterial vaccines[8, 9, 11, 34] or to the successful use of combined prophylactics in the immunization of mice and man[2, 4, 1] It is felt that these findings though of great practical importance are not necessarily in conflict with the positive results cited but merely underscore the complexity of the phenomenon and its dependence on the variables mentioned before

In previous work from our laboratory it was shown that guinea pigs which readily made satisfactory amounts of antibody in response to the injection of a standard amount of rabbit gamma globulin (RGG) made considerably less antibody against this antigen if they were concurrently injected with the total globulin fraction of rabbit serum or with hemocyanin Rabbit serum albumin was shown to be ineffective as a competitor It was also demonstrated that a dose of hemocyanin which was too small to cause competition when injected together with RGG into previously untreated animals caused a significant reduction in the response to RGG when injected with RGG into guinea pigs which had been sensitized to hemocyanin some time before

Currently we are trying to simplify the approach to a systematic study of CA through attempts to reduce the number of variables that enter into the problem Mice from highly inbred strains are used in order to reduce the variability in the immune response to various antigens which derives from genetic factors We also are using purified soluble antigens so that all the precipitating antibody produced against each of the antigens may be measured with some degree of accuracy Results obtained thus far are entirely preliminary in nature

Shown in Table II are the results of injection into DBA mice of purified hemocyanin from *Busycon canaliculatum* or of repeatedly recrystallized horse spleen ferritin[20] or of a mixture of the two in the ratio of 1 2 (nitrogen) It may be seen that the antibody formed against ferritin was significantly less in mice belonging to the experimental group (FH) than it was in mice of the control group (F) The response to hemocyanin among the experimental mice (FH) was not significantly different from that of controls (H) In this and the subsequent experiment the dose of each antigen given was selected so that the antibody response against each would be of approximately the same order of magnitude In each instance this dose was considerably less than that which was known to give a maximal antibody response under the experimental conditions employed that is after a single intraperitoneal injection of the antigen emulsified in incomplete Freund s adjuvant

TABLE II Antibody Formed in Response to Hemocyanin and Ferritin *

Group	Antigen	Ferritin Titers† (n)			
		3	6	11	14 Wks
F	Ferritin	8 5 ± 76	11 8 ± 71	13 0 ± 40	13 7 ± 1 2?
FH	Ferritin + Hemocyanin	6 3 ± 62	8 8 ± 69	9 0 ± 1 0	10 1 ± 1 01
	t	2 25	3 03	3 70	2 28
	Degrees of freedom	20	18	16	16
	Significance‡	+	++	++	+
		Hemocyanin Titers† (n)			
FH	Ferritin + Hemocyanin	7 4 ± 33	9 9 ± 27		9 9 ± 38
H	Hemocyanin	7 4 ± 43	9 5 ± 34		11 0 ± 19
	t	0	0 95		1 25
	Degrees of freedom	20	17		16
	Significance‡	—	—		—

* Female DBA mice injected once intraperitoneally with antigen in Freund's adjuvant without acid fast bacilli Hemocyanin 8γN Ferritin 15γN

† Mean and standard error of tanned cell titers Titer = 100 × 2^n

‡ ++ P < 0 01 + P < 0 05 — P > 0 05

Table III contains a summary of data obtained from an experiment in which DBA mice were injected with bovine gamma globulin (BGG) and ferritin The ratio of the doses of the two antigens (nitrogen) was 10 1 The upper half of the table shows that the injection of ferritin (5γ N) two weeks before that of BGG (48γ N) resulted in a significantly decreased antibody formation against BGG (Group 3) When the interval between the preliminary injection of ferritin and the subsequent immunization with BGG was extended to 4 6 or 8 weeks (Groups 4 5 and 6) the antibody production against BGG was initially retarded but it reached the level of antibody made by controls (Group 1) within 8 weeks after injection The mice in Group which had been injected with both antigens simultaneously showed a transitory lag in their antibody production against BGG

The lower half of the table shows that injection of BGG 4 to 8 weeks after that of ferritin had no measurable effect on the antibody production against ferritin The injection of BGG simultaneously with (Group 2) or 2 weeks after that of ferritin (Group 3) caused antibody production against ferritin to fall behind that observed in controls (Group 7) Since animals in Group 3 and possibly also those in Group 2 made less than the expected amount of antibody against BGG as well it would appear

TABLE III ANTIBODY RESPONSE TO FERRITIN AND BOVINE GAMMA GLOBULIN *

Group	Sex	Time of Injection of Ferritin	BGG Titers† (n) 4	6	8	10 Wks
1	M	BGG only	7 1 ± 3,	8 9 ± 46	9 6 ± 67	9 0 ± 86
1	F	BGG only	8 2 ± 39	9 5 ± 55	9 8 ± 75	9 6 ± 77
2	M	Simultaneous	(6 5 ± 24)‡	7 3 ± 40	7 8 ± 56	(7 8 ± 40)
3	M	2 wks prior	4 5 ± 41	5 8 ± 64	6 1 ± 64	4 6 ± 81
4	F	4 wks prior	5 8 ± 58	6 7 ± 48	(8 9 ± 61)	(9 0 ± 68)
5	M	6 wks prior	5 4 ± 69	6 2 ± 62	(8 5 ± 83)	
6	M	8 wks prior	5 8 ± 54	7 0 ± 50	(8 1 ± 67)	
		Time of Injection of BGG	**Ferritin Titers† (n)**			
2	M	Simultaneous	7 5 ± 48	8 1 ± 0 50	8 0 ± 49	7 8 ± 62
3	M	2 wks later	(7 2 ± 1 03)	7 6 ± 1 02	7 ‹ ± 89	7 8 ± 1 10
4	F	4 wks later		(10 8 ± 69)	(9 3 ± 47)	(10 2 ± 96)
5	M	6 wks later			(11 8 ± 66)	(11 6 ± 6)
6	M	8 wks later				(10 0 ± 98)
7	M	Ferritin only	9 7 ± 94	10 4 ± 65	10 4 ± 67	10 7 ± 85
7	F	Ferritin only	9 8 ± 65	10 8 ± 64	11 2 ± 69	10 9 ± 74

DBA mice injected once intraperitoneally with antigen in Freund s adjuvant without acid fast bacilli Bovine gamma globulin 48 γN Ferritin 5 γN

† Mean and standard error of tanned cell titers Titer = 100 × 2^n

‡ Parentheses around a pair of figures indicate that the mean does not differ significantly from that of control

that CA in this instance was reciprocal This peculiar finding requires further study especially aimed at exclusion of the possibility that an immune response to a third antigen such as bacteria causing an inapparent infection might have been involved In these two groups the simultaneous injection of both antigens appeared to affect more markedly the antibody response against the antigen given in the smaller amount (ferritin) where as injection of ferritin . weeks earlier resulted in a greater reduction of the antibody response against BGG than in that directed against ferritin

The mechanism by which introduction of one antigen interferes with the production of antibody against a second antigen remains obscure The analogy of competition of antigens with the interference phenome non in certain viral infections is striking but sheds no light on the subject Appeal to the ' dominance of one antigen over another merely restates the observation without adding to its explanation [14] In view of the meagerness of data on CA and because of our incomplete under standing of antibody formation any discussion of the subject falls of necessity into the realm of speculation

It appears that CA affects an early stage of antibody formation and that its intensity is related to that of the antigenic stimulus exerted by the *competing antigen*. The *formation of circulating antibody* specific for the latter is an indirect and not necessarily comprehensive measure of the magnitude of this stimulus.

There is considerable evidence to suggest that antibody synthesis involves *de novo* formation of immune globulin from some simple precursors, presumably from amino acids. It is conceivable, therefore, that CA may result from the demands on a common pool of raw materials. Some instances of CA may well arise from this cause, especially under circumstances in which the antigenic stimulus of the competing antigen is overwhelming. Tests of this possibility are within the reach of experimental tools and methods now available.

More appealing is the possibility that CA affects one of the links in the chain of cellular differentiation and proliferation which occurs during the adaptive and productive phases of antibody formation. Here too competition for raw materials from a common pool (which need not be confined to amino acids) could conceivably lead to CA. However, considering past failures in attempts to demonstrate linked antibodies—that is, antibodies reactive with more than one unrelated antigen—it seems possible that cells once committed to the production of antibody against one antigen may fail to respond to a second antigen and that CA may thus arise from a competition for cells.

At present there appears to be no answer to the question why, in the most common manifestation of CA, one antigen appears to enjoy a selective advantage over another. It seems fairly clear from the evidence on hand that a simple quantitative excess of one antigen over another does not insure occurrence of CA. The answer will not be forthcoming until more is known about the attributes of "immunogenicity" and the mechanism of antibody production itself. In the meanwhile it is hoped that further study of CA may reveal some facets of these problems which are not readily accessible by other approaches.

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DESIGNATED DISCUSSION

FELIX HAUROWITZ (Bloomington, Indiana): The organizers of this symposium asked me to present my philosophy on immunological unresponsiveness.* It may seem difficult at first sight to reconcile this phenomenon with theories on the mechanism of antibody formation[3, 11] and of protein formation in general.[10] The antigen molecule, according to these theories, interferes at the sites of antibody formation with the second phase of protein synthesis, with the conversion of the newly formed long peptide chain into a folded globular gamma globulin molecule whose shape is complementary to that of the determinant group of the antigen. It is not easy to understand how the same antigen molecule in immunological unresponsiveness should prevent antibody formation. An attempt will be made in this discussion to reconcile the two apparently contradictory actions of the antigen.

We have to remember first of all that the half life of the antibody molecules is in general shorter than that of the antigen. In rabbits the half life of the circulating antibody is approximately 5 to 6 days. The half life of tissue bound antigen, after the initial rapid elimination from the circulation, is at least 10 days. Six to nine months after a single injection we find in the spleen and liver an average of hundreds of antigen molecules per cell.[6, 12, 13] If large amounts of antigen are injected repeatedly, the amount of tissue bound antigen may become so high that all of the newly formed antibody is neutralized and converted into tissue bound antigen antibody complexes. Since 50 per cent of the circulating antibody in rabbits is regenerated in 5 to 6 days, the amount of the antigen required for complete neutralization of all of the antibody is theoretically equal to the amount of antigen which is formed within a period of two half lives, that is, 10 to 12 days. If we assume that the total amount of circulating antibody in the rabbit is approximately 0.5 Gm. per kilogram of body weight and that the antibody antigen weight ratio at neutralization is approximately 5, the amount of antigen required for the neutralization of all of the newly formed antibody would be approximately 100 mg. per kilogram of body weight. In practice much larger amounts of antigen are necessary to produce long lasting unresponsiveness, because some of the antigen molecules may be inaccessible or masked by combination with other cellular components, others may be catabolized, although the rate of catabolism seems to be lower for antigen than for antibody molecules.

* Support of the experimental work by contracts of Indiana University with the U.S. Atomic Energy Commission and the Office of Naval Research and by research grants from the U.S. Public Health Service and the National Science Foundation is gratefully acknowledged.

Since an excess of antigen is essential for this type of unresponsiveness the period of unresponsiveness in adult animals is extended if the amount of injected antigen is increased [14] It is also understandable that permanent tolerance is observed only after the injection of antigens which can either multiply such as viruses or grow like inoculated tissues All these phenomena indicate that unresponsiveness is caused by the persistence of an excess of antigen [1, 15] which traps newly formed antibody causes the formation of intracellular antigen antibody complexes and thereby prevents the passage of antibody into the circulation

Our own experiments give some evidence for the presence of antigen antibody complexes We find that isotopically labeled iodo- or azo protein antigens penetrate very rapidly into the intracellular granules of the cells of spleen liver bone marrow and other organs [12, 13] If this antigen were free it would be found in the intracellular fluids Evidently it is bound intracellularly possibly by nonspecific combination with other cellular constituents [8, 9] Trace labeled heterologous serum proteins behave quite differently They circulate for several days in the blood plasma of the injected animal and are then rapidly eliminated when antibody formation begins At the same time we observe an *absolute* increase in the antigen content of the tissues as shown by Figure 1 [6] This increase in tissue bound antigen must be due to the deposition of insoluble antigen antibody complexes

We do not know whether the antigen in these complexes is present as unchanged antigen or whether it has been degraded to fragments which are still able to combine with the antibody [9] Whatever the state of the antigen is it still contains its determinant binding group which in some of our experiments was labeled by radioactive isotopes The objection may be raised that Coons *et al* [5] using fluorescent antibody have not been able to detect antigen in the antibody containing cells However antigen antibody complexes in contrast to free antigen may not always combine with added fluorescent antibody

If very large amounts of antigen are injected only part of the tissue bound antigen can consist of antigen antibody complexes Another part of the antigen is bound nonspecifically to other cellular substances such as proteins nucleic acids lipids or polysaccharides [8] This tissue bound antigen may act as a trap for newly formed antibody molecules and prevent their passage into the blood On the other hand the antigen antibody complexes may be able to combine with small amounts of free antigen and to prevent thereby their appearance in the circulation This reaction can be represented by the equation $AgAb_n + Ag \rightarrow AgAb_{n-1} + AgAb$ where Ag = antigen Ab = antibody and AgAb a small antigen antibody complex Obviously the AgAb complex can react in this manner only with molecules of the homologous antigen not with other types of antigen

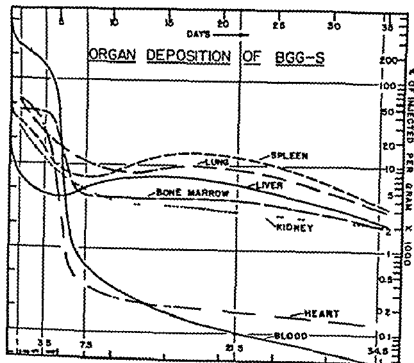


FIGURE 1 Organ distribution of beef serum gamma globulin labeled by traces of S^{35} sulfanilic acid in the organs of rabbits injected intravenously with 15 30 mg of the antigen per kilogram of body weight

This may explain the high specificity of the immunological unresponsiveness and the ability of the organism to form other types of antibody

The crucial problem in all forms of unresponsiveness is the question whether the small amounts of tissue bound antigen or of antigen antibody complexes are significant and whether they are essential for the immunological phenomena Labeling the antigen with radioactive isotopes allows us to discover a few micrograms of antigen in 100 or more grams of tissue or organ It is difficult however to decide whether the small amount of radioactive material is still present as the unchanged determinant group of the antigen molecule and whether the traces of antigen found are significant Only in few instances it has been shown that the radioactive material is identical with the injected material In many other instances also this may be true although it has not yet been proved The objection has been raised that antigen molecules cannot persist over long periods of time in the continuously changing population of cells We have to remember however that pigments introduced by the process of tattooing persist for many years in the same location in spite of growth and changes in the cell population of their environment

In evaluating the small amounts of persisting antigen we have to keep in mind that each molecule of diphtheria toxoid can give rise to about one million antitoxin molecules in the course of three weeks[4] Hence even very small amounts of tissue bound antigen may be highly significant and may give rise to the production of large amounts of antibody The well known immunological response is evidently due to the production of a large amount of antibody which neutralizes all of the tissue bound antigen and then penetrates into the circulation It is tempting to assume that immunological unresponsiveness results when the amount of antibody formed is so small that all of it is neutralized by the tissue bound antigen and converted into antigen antibody complexes

It is difficult to understand the long lasting tolerance in animals which were injected with small amounts of an antigen during their fetal life Since the fetus has not the ability to form antibodies the injected antigen forms a large excess It may also be in excess at a time when antibody formation normally begins Later however the small amount of antigen injected into the fetus may hardly be sufficient to form an excess Its long lasting action which causes unresponsiveness may be due to the particular properties of the fetal tissues in which the rate of anabolic processes exceeds by far the rate of tissue breakdown It is imaginable that a larger portion of the injected antigen molecules is built permanently into the growing cells The half life of these specifically bound antigen molecules may be much longer than that of antigen molecules which are injected into adult animals They may therefore persist in the fetal and later in the adult organism in spite of dilution by growth and multiplication of the cells

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PHILIP Y PATERSON (Bethesda Maryland and New York New York) There are many reasons why an animal may fail to respond to antigenic stimulation This morning Dr Chase and Dr Weigle have described particular forms of immunological unresponsiveness Dr Chase has described the specific suppression of antibody formation against certain allergenic chemicals induced in adult guinea pigs by feeding of these chemicals Dr Weigle has reported inhibition of antibody production against protein antigen in rabbits previously injected with large amounts of the antigen during early neonatal life In both of these studies lymph nodes from unresponsive donor animals have been found to give no demonstrable response to the antigen in question after transfer to recipient animals Thus failure of antibody production appears to account for these forms of unresponsiveness

Dr Stark has presented studies of another example of altered immune response called immunological paralysis This phenomenon as defined by Felton refers to the failure of mice to respond to an immunogenic dose (0 0005 mg) of pneumococcal polysaccharide following prior injection of relatively large doses (0 5–0 005 mg) of polysaccharide of the same type It is presumed that there is no failure of antipolysaccharide antibody production in immunological paralysis Constant absorption of antibody by excess tissue fixed polysaccharide is assumed to occur Metabolism of antibody is believed to permit persisting tissue polysaccharide to absorb additional antibody The data presented by Dr Stark appear to provide additional support for this explanation of immunological paralysis

It is conceivable that immunological paralysis is similar to those forms of immunological unresponsiveness described by Dr Chase and Dr Weigle Is it established that mice injected with relatively large doses of

pneumococcal polysaccharide do elaborate antipolysaccharide antibody In this regard Felton *et al* reported that mice paralyzed by injection of Type I polysaccharide and subsequently injected with formalin treated homologous pneumococci later survived challenge with virulent Type I pneumococci However comparable results were not obtained using Type II and Type III polysaccharide and pneumococci Experiments similar to those described by Dr Chase and Dr Weigle might be employed to determine whether mice exhibiting immunological paralysis do or do not elaborate antibody viz transfer of lymph nodes from paralyzed mice to normal mice Conversely it would be desirable to know whether lymph nodes from mice injected with immunogenic doses of polysaccharide can or can not confer protection upon paralyzed recipient mice

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IX

Tolerance and Rejection of Tissue

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35

Transplantation Immunity and Hypersensitivity

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The transplantation of living tissues from one individual to another belonging to the same species (homografts) usually ends in the destruction of the grafts The processes involved in graft rejection have recently been extensively reviewed [9, 14, 20, 25] Briefly the so called homograft reaction is an immunological response to substances present in the graft but not in the recipient or host when a situation arises in which both have the *same* tissue antigens as does happen when donor and recipient are identical twins the homograft reaction is conspicuous by its absence The antigens concerned are genetically determined by the histocompatibility genes which appear to segregate as Mendelian factors Prolonged inbreeding has yielded mouse guinea pig and rat strains the members of which are so alike that tissue grafts may be exchanged between them with impunity and such inbred strains have provided a vital tool for studies on the transplantation of tissues Needless to say tissue grafting from one part of the body to another part of the same individual (autografts) will not create immunological problems other than those connected with specific tissue or organ specific antigens

There can no longer be the slightest doubt that the homograft reaction is a specific immunological response However largely because circulating antibodies have been curiously reluctant to reveal themselves much of the evidence is circumstantial rather than direct The really central fact of tissue transplantation is that a state of immunity (or sensitivity) comes into being once an animal or human being has reacted against tissue homografts a second graft from the same donor or donor strain is therefore destroyed more rapidly than the first Immunity against normal tissues such as skin can be transferred to other individuals with the regional lymph node and spleen cells of immune donors [4, 22, 23] but not – in ordinary circumstances – with their blood serum [3] There is however the recent report by Stetson and Demopoulus [27] that immunity against skin homografts can be passively transferred with immune sera provided that Freund s adjuvant is used is the immunization of the serum donors There have been reports of protective [16] and cytotoxic [25] antisera

but these have in the main concerned neoplastic (leukotic) rather than normal tissues On the other hand the role played by *cells* in homologous transplantation immunity has been further emphasized by the experiments of Algire Weaver and Prehn[1,2] with diffusion chambers and evidence has recently been reported for the existence of antibodies in or upon the surfaces of immune lymphoid cells

The homograft reaction then is a specific immunological response leading to a systemic state of immunization If account is taken of the facts that (*a*) the reaction is accompanied by the infiltration into the graft of mononuclear cells (*b*) it is susceptible to interference with cortisone and its related compounds but not with the antihistamines and (*c*) immunity can be transferred with living immune cells but not in ordinary circumstances with serum it will hardly surprise that it has been fashionable in recent years to draw an analogy between transplantation immunity and certain delayed sensitivity reactions such as those elicited by tuberculin and some chemical compounds[4, 9, 10, 1, 18, 19, 3, 24] The experiments to be described carry this analogy one important step further for they provide direct support for the concept that transplantation immunity is essentially a very special state of hypersensitivity It will be shown that in guinea pigs a delayed skin reaction is elicited when antigenic tissue extracts are injected intradermally into specifically sensitized animals and that sensitivity can be transferred with living lymphoid cells It must be stressed that these experiments which have been carried out with the collaboration of Miss Jean Brown and Professor P B Medawar are still in progress at the time of writing and that much analytical work remains to be done

Materials and Methods

Most of the animals used were taken from a heterogeneous (noninbred) collection of albino guinea pigs obtained from commercial breeders In addition guinea pigs of a highly inbred strain (Heston by origin) maintained by us were used in certain special experiments skin grafting tests[6] having previously confirmed that these animals were entirely compatible

Preparation of Antigenic Tissue Extracts

The spleen of the antigen donor was excised (mean weight 0 97 Gm) and pressed through a metal sieve into 7–10 ml of normal saline (0 15 M) The resulting suspension was successively washed in normal saline dilute saline (0 05 M) and distilled water and then dispersed with the aid of a piston blender in about 8 ml of water The preparation was exposed for 1 minute to ultrasonic irradiation (for details see reference 6) and spun at 2000 g for 10 minutes the sediment being rejected The supernatant had

its sodium chloride content restored to 0 15 M and the resulting precipitate was removed by centrifugation again at 000 g for 10 minutes The supernatant was then spun at 5 000 g for 1 hour following which it was discarded and the sediment resuspended in a small volume of a suitable physiological solution The volume was then adjusted so that the sediment obtained from 1 Gm wet weight of spleen was contained in 1 ml of the suspension (strength = 1 1) Satisfactory dispersal was ensured by the use of a hand operated piston blender but the final preparation was brown gray strongly opalescent and particulate Further details of the extraction procedure may be found elsewhere[7] The great advantage of this method of preparation is that the antigenic material can be concentrated and to some degree purified although its color (when prepared from spleen) is certainly an undesirable feature from the point of view of skin reactions it is not in any sense a fatal handicap Clear aqueous solutions[8] have also been successfully used but being by necessity more dilute they have tended to produce somewhat feebler effects

Preparation of Lymph Node Cell Suspensions

Immune lymph node cells were obtained from guinea pigs which had previously reacted against a single set of 4 skin grafts from the prospective antigen donor The use of skin grafts in the immunization procedure was largely a matter of convenience since they could be removed without in any way prejudicing the donor s survival Other tissues could in theory be used with equal success but skin grafts have the additional advantage of providing quantitative information of the degree of incompatibility between donor and host On the destruction of the grafts (usually accomplished 10 or 11 days after transplantation to the right thoracic wall) the large regional (axillary) lymph node was excised and teased out in about 0 5 ml of Ringer phosphate (pH 7 4) The connective tissue fragments were hooked out and the suspension allowed to stand in a narrow tube for 30 seconds to allow the larger clumps to settle out The cells in the supernatant were counted and the volume of the suspension adjusted so that 1 ml contained 80 to 100 million cells This method of preparation was adopted in order to minimize possible injury to the cells by centrifugation and redispersal The suspensions were almost free from red blood cells Nonimmune lymph node cells were prepared in an identical manner the lymph nodes having been removed from normal guinea pigs

Injection of Guinea Pigs

All injections whether of tissue extracts or cell suspensions were made intradermally into the flanks of the recipients with a No 30 gauge needle mounted on a lubricated tuberculin syringe Although all injections were generally intradermal there was some variation in the planes of injection

some being more superficial than others This did not materially affect the results

Dosages

The volume of tissue extract per injection site was 0 05 ml in the majority of experiments but more recently we have been injecting 0 1 ml Each flank usually received several aliquots of extract Although the standard dilution of the extract was 1 2 or 1 4 higher dilutions of up to 1 16 have been tested

When transferring sensitivity with lymph node cells 4 to 6 million cells contained in 0 05 ml were injected per site but again more dilute suspensions (down to 500 000 cells/0 05 ml) have been tested

Appraisal of Results

The skin reactions were scored according to two criteria (*a*) the degree of inflammation as judged by edema reddening and necrosis and (*b*) the area of inflammation For example a strongly developed reaction scored as + + + + + was a raised and bright red area of about 12 mm diameter with or without limited central necrosis at the other end of the scale an extremely feeble reaction (as sometimes encountered in the controls) consisted of a tiny area of only just perceptible redness (± or + —) Definitive readings were taken when the reactions were maximal that is between 24 and 36 hours but additional readings were taken at regular intervals

DESIGN OF EXPERIMENTS AND RESULTS

Skin Reaction Elicited by Tissue Extracts

The initial problem was to discover whether the injection of tissue extracts into specifically immunized guinea pigs would lead to a skin reaction of any kind Because the method of extraction was identical with that used for the preparation of highly antigenic tissue extracts in the mouse [7] it could reasonably be presumed that the guinea pig extracts also contained antigenic matter For comparison normal nonimmune animals were injected with the same extracts (Figure 1) It will be seen from Table I that all the 16 immune guinea pigs gave either a moderate or strong skin reaction the 13 normal control animals on the other hand reacted in only the feeblest possible manner or not at all

The reaction in the immune animals had all the elements of a typical delayed response Firstly it was delayed the injection sites generally showed visible inflammatory signs 5 to 8 hours after injection and the reaction s peak was reached between 24 and 36 hours At this stage the sites were edematous and very red and measured up to 12 mm in diam

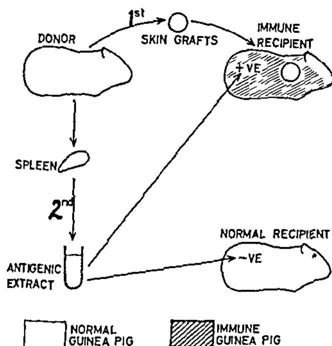


FIGURE 1 Illustrating the injection of homologous spleen antigenic extract into (*a*) an immune guinea pig and (*b*) a normal guinea pig

TABLE 1 SUMMARY OF BASIC EXPERIMENTS IN GUINEA PIGS*

Number of Recipients	Status of Recipients	Material Injected	Dose	Result (Strength of Skin Reaction)
16	Immune	Antigenic extracts	0.05 → 0.1 ml of 1:1 → 1:4	+++ → > +++++
13	Normal	Antigenic extracts	0.05 → 0.1 ml of 1:1 → 1:4	0 → ±
9	Skin graft donors	Immune cells	4 to 6 million in 0.05 ml	Generally > +++++
11	Normal guinea pigs	Normal cells	4 to 6 million in 0.0 ml	0 → +
3	Skin graft donors	Immune serum	0.05 → 0.1 ml	0

* All injections of homologous extracts or cells were intradermal. Skin reactions scored after 24 hours

eter The inflammatory symptoms gradually faded and were negligible on the third day Secondly the histological picture resembled very closely that described by Gell and Hinde [13] for the tuberculin reaction there was little tissue damage but a marked local infiltration of mononuclear cells Thirdly it proved possible to transfer this sensitivity with living immune lymphoid cells but up to now not with immune serum (see below)

In order to make even more certain that the skin reactions were not due to nonimmunological factors nor to an immunity directed against anything except the homologous antigens experiments making use of inbred animals were carried out (Figure) Briefly the reactivity of inbred recipients which had been sensitized with grafts from noninbred donors was tested with (*a*) extracts from the specific (noninbred) donors and (*b*) extracts from other inbred animals Whereas the former elicited strong skin reactions the latter did not At the same time the potential antigenic potency of the inbred extracts was demonstrated in noninbred recipients which had previously been sensitized with skin grafts from inbred animals here good reactions were obtained The skin reactions could therefore hardly be ascribed to other than specific immunological causes

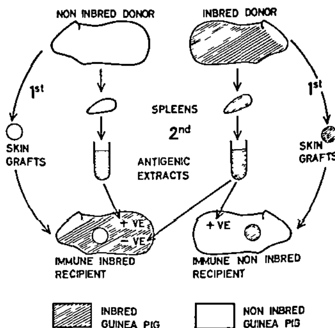


FIGURE 2 Illustrating the injection of homologous spleen antigenic extracts from inbred and non inbred donors into immune inbred and non inbred guinea pigs

The strongest reactions were obtained with the more concentrated extracts but dilutions of down to 1 : 8 gave perceptible skin reactions.

Transfer of Sensitivity with Immune Lymph Node Cells

It is well known that tuberculin sensitivity and sensitivity to simple chemical compounds can be transferred to normal animals with lymph node cells or peritoneal exudates injected via a systemic route [11, ... 21]. Transplantation immunity too can be transferred by exactly analogous methods with the regional nodes of immunized animals [4]. It was therefore natural to investigate the possibility that the state of hypersensitivity responsible for the delayed skin reaction in our experiments might be transferable with cells from regional immune lymph nodes. Because the antigens with which we were concerned are homograft antigens and therefore generally distributed throughout the tissues of normal animals, this transfer was accomplished by a novel and (as it turned out) delicate method (Figure 3). Guinea pigs were immunized by the transplantation of skin grafts and cell suspensions were then prepared from the regional (axillary) nodes. The cells were injected intradermally, that is locally instead of systemically, into the *skin graft donors*, that is the same animals

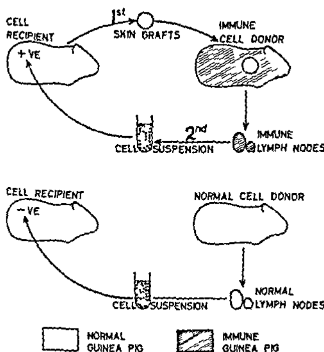


FIGURE 3 Illustrating the intradermal injection of immune and normal lymph node cells into homologous guinea pigs using separate recipients

against whose tissues the cells had been immunized As a control normal nonsensitized cells were injected into other normal animals For stricter comparison a number of experiments were designed in which the skin graft donors received immune as well as normal cells (Figure 4) Without exception the injection of immune cells brought about a well developed delayed skin reaction resembling in many respects that elicited by tissue

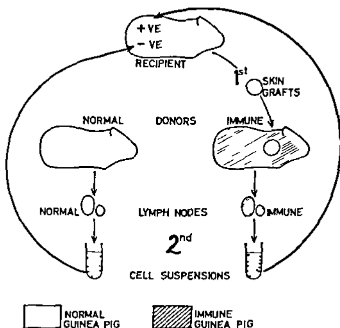


FIGURE 4 Illustrating the intradermal injection of immune and normal lymph node cells into the same guinea pig

extracts in sensitized recipients (Table I) By contrast the injection of equal numbers of nonsensitized cells generally yielded negative results although in some recipients very slight inflammatory symptoms wer observed These however were far too feeble to obscure interpretation of the data

Although the skin reaction resulting from the injection of immune cells into antigenic recipients appeared similar to that elicited by tissue extracts in immune recipients one or two differences were evident The former reaction tended to reach its peak a little later (36 to 48 hours) was nearly always of more dramatic intensity and faded away more slowly (3 to 6 days)

As before special experiments involving inbred animals were carried out (Figure 5) An inbred guinea pig was immunized with skin grafts from a noninbred animal which was subsequently injected with (*a*) the specifi

cells immune cells and (*b*) cells from another inbred animal According to expectation the former elicited a strong reaction while the latter did not nor did the immune inbred cells elicit a reaction when injected into the skin of yet another inbred recipient Here then was clear evidence of the specific immunological nature of the reaction brought about by the transfer of immune cells — evidence which was fully corroborated by

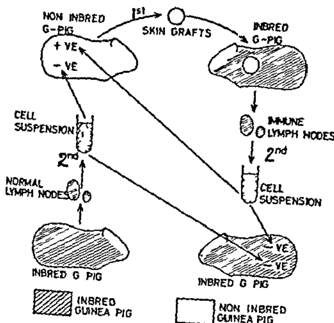


FIGURE 5 Injection into (*a*) an inbred guinea pig and (*b*) a non inbred guinea pig of cells taken from the lymph nodes of (*a*) a normal inbred guinea pig and (*b*) an inbred guinea pig which has been sensitized against the non inbred animal

experiments in which lymph node cells from an immunized guinea pig were injected back into its own skin (Figure 6) again no reaction could be observed

Our attempts to bring about a skin reaction by the injection of immune sera into antigenic recipients have so far failed (Table I) but it must be stressed that these experiments are as yet in their infancy few experiments have been performed so far and the effect of hyper immune sera remains to be investigated

Experiments in Mice and Rabbits

We have been quite unable to reveal a comparable phenomenon in mice Out of 18 immune mice tested with tissue extract of known antigenic

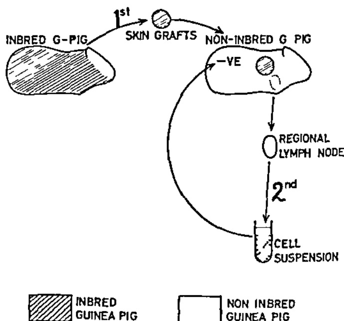


FIGURE 6 Illustrating the intradermal injection of immune autologous guinea pig lymph node cells

potency none produced a significant reaction no matter whether the extracts were injected into the body skin or foot pad Several strain combinations have been tested viz A → CBA CBA → A and A → C57 It must be admitted that the injections were hardly intradermal because of the thinness of the skin the extracts were inevitably injected subdermally (though above the panniculus carnosus) Attempts to elicit skin reactions by the injection of immune lymphoid cells into antigenic recipients were equally unsuccessful all of 10 recipients (strain combinations CBA → A and C57 → A) remained entirely negative when injected in the foot pad or body skin nor did adrenalectomy of the recipients in any way affect the course of such experiments

It may be mentioned in passing that preliminary results in rabbits indicate that antigenic extracts will bring about only a feeble inflammatory response in immune animals However the transfer of cells in o antigenic recipients has resulted in very powerful reactions ending in extensive tissue necrosis

COMMENTS AND CONCLUSIONS

The fact that a skin reaction of the delayed type can be brought about by the intradermal injection of homologous tissue extracts into immune guinea pigs provides direct evidence of the close immunological affinity

between transplantation immunity and certain delayed allergic responses such as tuberculin sensitivity and drug allergy In keeping with this concept is our failure to demonstrate skin sensitivity in the mouse — an animal well known to be an extremely unfavorable subject so far as delayed allergic responses are concerned Homologous transplantation immunity may therefore be regarded as a state of hypersensitivity and the second set response (i e the accelerated rejection of a second graft from the same donor) as a direct expression of this It just so happens that the guinea pig is endowed with a unique talent for revealing antigen antibody reactions by giving a characteristic inflammatory response provided that the antigenic stimulus is administered in a suitable physical form and by the intradermal route

As already stated the justification for regarding the skin reaction described here as a typical delayed reaction rests on four lines of evidence some of which are at present more firmly established than others Firstly the timing of the reaction follows the classical pattern of delayed allergic responses Secondly the cellular aspects of the reaction resemble those described for the tuberculin reaction[3] Thirdly the admittedly flimsy evidence available at the time of writing does not encourage the belief that sensitivity is transferable with immune sera And fourthly there is ample evidence that the sensitivity elicited by homograft rejection can be transferred to normal guinea pigs with cells from the regional lymph nodes of sensitized animals This last point requires further comment

The transfer of tuberculin sensitivity and sensitivity to such chemicals as picryl chloride has been achieved by the systemic injection of sensitized lymph node or peritoneal exudate cells into normal guinea pigs followed by skin challenge with the appropriate antigen The transfer of sensitivity in our own experiments has been brought about in a somewhat different manner because of the special nature of the antigens which — being cellular — occur naturally throughout the tissues of normal guinea pigs This has enabled us to transfer sensitivity locally rather than systemically and therefore to make the test system unusually delicate The transfer of sensitized lymph node cells into the skin of a guinea pig with the correct antigenic configuration (in practice the donor of the skin grafts used for sensitization) is not likely to differ fundamentally from a systemic transfer here the antigenic stimulus is immediately and automatically provided by the cells of the skin

In guinea pigs the skin reaction produced by the transfer of sensitized cells is only quantitatively distinguishable from that elicited by tissue extracts in sensitized animals it takes a little longer to reach its peak and its intensity is nearly always greater Both these differences may be attributed to the same cause namely that a relatively large number of sensitized cells are crowded into a small volume and surrounded by an

indefinitely large supply of antigenic material which as inflammation gets under way, is even further reinforced by the arrival of host cells In theory once having established a miniature 'lymph node the donor cells could go on reacting indefinitely in practice they would be expected to be overcome by a homograft reaction directed against them by the host – an expectation which is borne out by the observation that the reaction fades away between the third and sixth day following transfer It might well be argued that the reaction is altogether due to a homograft reaction against the transferred homologous cells but this is hardly justified in view of the relatively rapid onset of the reaction. Moreover a significant skin reaction has never been observed after the injection of *normal* lymph node cells into normal recipients – a finding which completely rules out such an interpretation Until evidence is discovered to the contrary it may therefore be taken for granted that the reaction elicited by sensitized cells injected into the homograft donor has the same basic mechanism (i e reaction between antigens and cellular antibodies) as that elicited by the injection of antigenic extracts into sensitized animals Indeed it may be regarded as a passive reverse reaction in the sense in which this term is understood in other immunological systems – passive because the cells are transferred passively, and reverse because the antibody carrying cells are injected into an antigenic environment rather than as is more usual the other way about

The above interpretation of the sensitivity phenomena could be further tested by an experiment in which sensitized (inbred) cells are injected into a neutral (also inbred) host immediately after being mixed with the specific antigenic extract (noninbred) Experiments of this kind are now in progress and have so far given encouraging result

It has been argued that the transfer of tuberculin sensitivity may result from the activation of the hosts own cells by the sensitized donor cells (for review see reference 8) The local passive reverse reaction described above seems to provide evidence against the concept of activation for it is difficult to imagine (*a*) that the cells present in normal skin should be susceptible to such activation and (*b*) that activation should occur with such rapidity In our system at least it may be accepted that the reaction is the direct consequence of an interaction between the donor cells and the host antigens This conclusion is entirely in agreement with recent evidence regarding the nature of the cellular transfer of tuberculin sensitivity [21]

The skin reactions uncovered by these experiments may become useful tools for further studies in the field of tissue transplantation and perhaps, in the field of general immunology The reaction elicited by locally administered antigenic extracts is about ten times more sensitive for the purpose of evaluating the antigenic potency of extracts than the

method employed up to now which is based on the accelerated destruction of second set homografts Expressed in terms of wet weight of spleen (the antigen source) the former can be produced by the extract from as little as 10 milligrams whereas the latter requires the injection of at least 100 Similarly the passive reverse reaction can be elicited by less than 1×10^8 sensitized cells — roughly one hundredth of the number required to transfer immunity in the mouse or guinea pig It is hoped to apply this new test system to the study of subcellular fractions obtained from the lymphoid cells of sensitized animals Its main drawback from the point of view of general immunology lies in the fact that a great many tissue antigens are involved and that the antigens are as yet chemically undefined

The possibility of desensitizing guinea pigs sensitized to homologous tissue antigens is now being investigated

ACKNOWLEDGMENTS

The collaborative nature of this work has already been referred to in the text We wish to thank Mr T Courtenay for his technical assistance

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The Role of Antibody in the Rejection of Homografts

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The basic phenomena of transplantation immunity are usually stated somewhat as follows: a skin homograft is initially well accepted and vascularized but after some days it is suddenly vigorously rejected; then, after a suitable interval of time, a second homograft applied to the same animal will be tolerated for a much shorter time. This formulation, although historically well established, is not quite the whole story. By varying the interval between application of the first and second grafts it can be shown that the fate of the second graft is very much dependent on the length of this interval. For example, after application of a 10 mm circular skin homograft on a rabbit's ear, second homografts applied during the first week exhibit progressively shorter survival and tend to be rejected at or about the same time as the first graft. There then follows a period of several days during which a second homograft will not be accepted at all. That is, it is not vascularized or healed in, and its relation to its host is chiefly a matter of the sutures that hold it in place. It can sometimes be lifted off from the graft bed even after several days without causing bleeding. We have demonstrated failure of vascularization by direct examination on the animal with the capillary microscope, by histologic examination, by injection of Evans Blue intravenously with failure of the dye to enter the graft, and by failure of the graft to bleed when pricked or incised. The graft eventually dies and dries up, and exhibits throughout all this time a blanched appearance which has suggested the term "white graft."[2] During this time the host may be said to be specifically "immune" in the classical sense that this population of foreign cells is simply not permitted to establish itself any more than streptococci can establish themselves in a specifically immune animal. This "transplantation immunity," as we believe it to be, has unaccountably received little attention in the transplantation literature, although it has been observed and described by Billingham, Brent, and Medawar[1] and by Rapaport and Converse.[2] It is short lived and is soon replaced by a state of affairs in which the animal, now evidently no longer immune, initially accepts and vascularizes the second homograft but rejects it in an accelerated fashion (Figure 1).

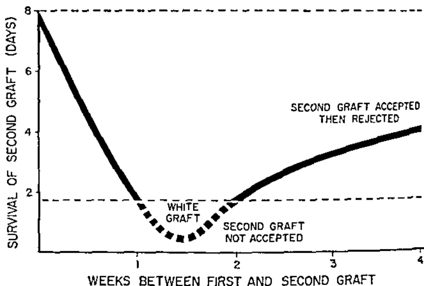


FIGURE 1 The fate of second homografts applied at various intervals after the first homograft Individual rabbits each received two skin homografts from the same donor The time interval between application of the first and second grafts is indicated on the abscissa and the survival time of the second homograft is shown on the ordinate The heavy line indicates the average survival time of the second grafts with the dotted portion representing the period of white graft immunity For reference purposes the upper horizontal dotted line shows the average survival time (8 days) of single skin homografts of this type in rabbits and the lower horizontal dotted line indicates the time (1 to 2 days) required for vascularization of a skin homograft

Second grafts applied during the first week after the first graft were initially vascularized then rejected together with the first graft When application of the second graft was delayed from 1 to 2 weeks after the first graft (until the time when the first graft was undergoing rejection) the second grafts were not vascularized and died without exhibiting hemorrhagic necrosis During the third and fourth weeks the application of second grafts was again followed by initial acceptance healing and vascularization with subsequent accelerated rejection

These facts can we think be readily explained in standard immunologic terms The introduction of an antigen into a normal animal classically has two easily demonstrable results the animal develops specific circulating antibodies and at the same time develops an equally specific capacity to react with an accelerated or secondary or anamnestic antibody response to a second antigenic stimulus Our working hypothesis is that the white graft immunity seen during the second week is due to circulating antibody produced in response to the first homograft while the accelerated rejection observed in the third and fourth weeks is the manifestation of a secondary or anamnestic response initiated by the second homograft A white graft reaction according to this hypothesis is the result of the reaction between a homograft and preformed circulating

antibody while the subsequent accelerated rejection reaction involves an active and anamnestic response to the second homograft following its initial acceptance and vascularization

If this hypothesis is correct it should be possible to passively transfer the "white graft" immunity with serum just as one can passively transfer antibacterial immunity with antibody-containing serum On the other hand it should *not* be possible to transfer the accelerated rejection phenomenon with serum since the capacity of an animal to exhibit a secondary response to an antigen cannot of course be transferred in this way

Our own experiments have been principally aimed at the production of white graft immunity and its transfer by serum In these experiments we have had no reason to assume that the application of a skin homograft is the most effective means of immunization for example transplantation immunity can be conveniently induced by the injection of suspensions of cells derived from the spleen or other organs We have followed the procedure of Voisin and Maurer [10] and incorporated spleen cell suspensions into an emulsion containing Freund's complete adjuvant mixture and injected the emulsion into the toe pads or foot pads of homologous mice or rabbits This technique is probably the best and certainly the quickest method of obtaining high titered antisera to conventional antigens Animals immunized in this way regularly show as might be expected specific white graft immunity With serum from such animals obtained 1 to 3 weeks after immunization this white graft immunity can be passively transferred either by intravenous injection of serum at or about the time of homografting or by local injection around the test and control homografts

Except for the suggestive experiments of Voisin and Maurer [9] serum transfer of skin homograft immunity has not been reported so far as we are aware There is however, an abundant literature on the passive transfer of immunity to tumor homografts With rare exceptions these successful experiments have involved leukotic tumors while immunity to orthotopic grafts of solid tumors is generally not transferable with serum It is possible that the effect of antibodies on homografts may be dependent on the accessibility of the antibodies to the cells of the graft and suspensions of homologous cells might thus be more quickly or severely damaged than solid grafts which are penetrated more slowly The characteristics of the immune response to homografts have recently been extensively reviewed by Gorer [6] and Medawar [7] and may be summarized as follows homografts of tumors and normal tissues are often followed by the appearance of circulating antibodies to various histocompatibility antigens In some cases tumor immunity can be passively transferred with serum containing these antibodies Tumor and skin homograft immunity

can also be passively or adoptively transferred with regional lymph node cells which continue to produce antigraft antibodies in their new host

Direct evidence that antibodies mediate tumor or normal tissue graft immunity is confined of course to those relatively few cases in which the immunity has been passively transferred with serum Extrapolation of those results to the general problem of transplantation immunity would surely be unwise at the present time On the other hand it should be pointed out that none of the objections usually raised to the antibody theory are serious and all must give way if passive serum transfer should become a reality For example the well known experiments of Weaver Algire and Prehn [11] with cell impermeable chambers do demonstrate that host cells somehow participate in graft rejection but these experiments do not answer the central question of whether antibody is *also* required as the basis of the specific immunity Streptococci survive nicely in immune serum and would not be expected to perish in a chamber inserted into the peritoneal cavity of an immune mouse unless host cells were also admitted It would of course be incorrect to conclude from such an experiment that streptococcal immunity was not due to specific antibodies and the same situation may obtain in the case of homografts Medawar[7] cites the experiments of Schinkel and Ferguson [9] who demonstrated skin graft rejection by normally agamma globulinemic fetal lambs as evidence against the participation of antibody in graft rejection This argument would surely have more weight had it actually been shown that the grafted lambs in fact failed to make antigraft antibodies or gamma globulin for the agamma globulinemic status of the normal fetal lamb may be a reflection of the efficiency with which nature has protected him from antigenic stimulation rather than an expression of immunologic unresponsiveness Certain it is that the agamma globulinemic human fails to develop transplantation immunity [4] as well as streptococcal immunity quite possibly for the same reason

The formation of plasma cells in the graft bed has been carefully studied by Darcy [3] who found a nice correlation between the time of appearance and numbers of plasma cells and the time of rejection of the graft These plasma cells may be presumed to be making antibodies against graft antigens though this has not yet been shown to be the case nor can it be *a priori* assumed that such antibodies play any role in the homograft reaction Experiments along these lines must probably await an elucidation of the nature of the graft antigens responsible for transplantation immunity However the participation of locally formed antibody in the homograft reaction would accord well with at least some of the facts. The graft bed and the regional lymph node appear to be the chief seats of immune response to homografts and the concentration of antibody there

would be expected to be far higher than in the animal as a whole or in his peripheral circulation Previous attempts at passive transfer of graft immunity using the serum of grafted animals may have failed for purely quantitative reasons

The term transplantation immunity dates back roughly a half century to a time when the basic phenomena of immunology were not so well defined as they are now [7] It may be that the application of this term to the accelerated rejection reaction has been an historical accident and consideration of the white graft type of reactivity as the actual immune state may be more in accord with the facts at hand After the waning of white graft immunity the capacity of the animal to exhibit an accelerated response to a second homograft may well be somehow related to delayed hypersensitivity Until the white graft phenomenon and the accelerated rejection phenomenon are more clearly understood however it would seem prudent to treat them separately

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*Acquired Resistance to Transferred Lymph Node Cells**

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Since the studies by Landsteiner and Chase [12] and by Chase [5] on the transfer of delayed hypersensitivity by cells there has been wide application of the technique of cell transfer to the study of several types of immunologic reactions [6, 7, 18, 19, 22]. In this laboratory the technique of lymph node cell transfer has been applied to the study of some aspects of the formation of humoral antibodies. These experiments have been done in the heterogeneous stocks of rabbits which are available, and the introduction of cells of one animal into the tissues of another, which, although of the same species, is not genetically identical with the first, has provided a situation for the study of immunologic reactions to cells of the type transferred. Data obtained in studies of some of these problems will be presented below.

The Lymph Node Cell Transfer Systems Employed

Transfer of Lymph Node Cells from Antigen-injected Donors

In the earlier studies of this series [9] the antigenic material, usually a suspension of *Shigella paradysenteriae*, was injected into the foot pads of rabbits and after several days the animals were sacrificed. Lymph nodes draining the sites of injection of the antigen were excised and teased, and the lymph node cells thus obtained were transferred to recipient rabbits. Sera of the recipient animals were subsequently examined for agglutinins to the organisms which had been injected into the donor animals. Agglutinins were found in such sera on the day after cell transfer, the titer rising until the third or fourth day after cell transfer and then declining gradually. This sequence is schematically shown in the left half of Figure 1.

On the basis of several types of evidence it was concluded that the appearance of agglutinins in the sera of recipients after the transfer of the lymph node cells could not be attributed either to the transfer of preformed antibody or to antibody formation by the tissues of the recipient.

* This study was supported by Research Grant H 869 of the National Heart Institute, National Institutes of Health, U.S. Public Health Service.

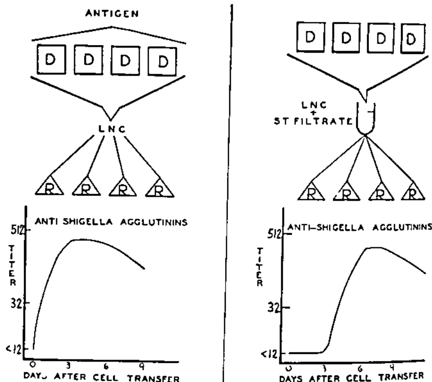


FIGURE 1 Diagrammatic representation of lymph node cell transfer with antigens of *Shigella paradysenteriae* In the type of experiment represented on the left Shigella were injected into the foot pads of the donor rabbits (D) Cells obtained from the draining lymph nod s 3 days later were transferred to recipient rabbits (R) The recipients were bled subsequently at regular intervals and their sera tested for anti Shigella agglutinins The general pattern of the titer of such agglutinins is shown in the curve on the left

In the type of experiment illustrated on the right the donor rabbits (D) were sacrificed and lymph node cells obtained from them were incubated *in vitro* with a filtrate of a trypsin treated suspension of *Shigella paradysenteriae* (ST filtrate) These cells were transferred to recipients (R) The typical pattern of appearance of anti Shigella agglutinins is shown in the curve on the right

animal as a result of antigen transferred in the cell suspension but rather that it was probably due to the transfer within the cells of a mechanism involved in the production of antibody [9] Certainly it was clear that antibody appeared only after the transfer of viable cells If the cells were frozen and thawed 3 times alternately in a dry ice and alcohol bath or irradiated in the ultraviolet range or exposed to iodoacetate at a concentration of 10^{-2} or 10^{-3} M or incubated at 37 C for 24 hours it was found that agglutinins did not appear in the usual pattern in the sera of recipients of cells so treated

Subsequently experiments were done in which the interval between the injection of antigen into the donor and the collection of its lymph node cells was progressively decreased [8] In such experiments a complementary relation was found to exist between the interval from the injection and sacrifice of the donor on one hand and the interval between cell transfer and the appearance of agglutinins in the recipient on the other These observations suggested that following the introduction of dysentery bacilli into the hind foot pads of rabbits the production of agglutinins to these organisms in the regional lymph node was a continuous process which if interrupted in the tissues of the donor could be continued in the tissues of a homologous recipient

The in Vitro Antigen incubation System

The results of experiments involving the progressive decrease of the interval between injection of antigen and sacrifice of the donor animals led to the observation that agglutinins could be found in the sera of recipients not only of cells which had had contact with the antigen in the tissues of the donor but also of cells allowed contact with the Shigella derived antigen only *in vitro* Subsequent studies were done largely in this system the experiments being carried out as follows Lymph node cells were obtained from uninjected donor rabbits (or from rabbits previously injected with a heterologous antigen in order to increase the yield of cells) incubated *in vitro* with a solution of antigenic material derived from Shigella washed and transferred to irradiated recipient rabbits [10] The pattern of these experiments is shown in the right half of Figure 1 and below this is shown the typical time curve of agglutinins to Shigella found in such experiments the appearance of agglutinins at a detectable level on the third or fourth day the attainment of a maximal titer on the sixth to eighth day and the gradual decline of the titer thereafter In this system as in the donor injection system injury to the transferred cells resulted in the failure of agglutinins to appear subsequently in the sera of the recipient rabbits

THE EFFECT OF PRE-INJECTION OF RABBIT LEUKOCYTES ON THE RESULTS OF LYMPH NODE CELL TRANSFER

The function of the transferred lymph node cells in relation to the production of the antibody found subsequently in the recipients sera is a matter of considerable interest This question was approached in a series of experiments designed at altering specifically the environment into which the lymph node cells were transferred by injecting the recipient rabbits prior to cell transfer with leukocytes from the donor rabbits In such experiments blood specimens were obtained from the prospective

donor rabbits and leukocytes obtained from these were pooled washed and injected into the prospective recipients On the day of cell transfer the donor rabbits were sacrificed Cells obtained from their lymph nodes were incubated with Shigella trypsin (ST) filtrate washed and transferred to the recipient rabbits The plan of these experiments is shown schematically in Figure

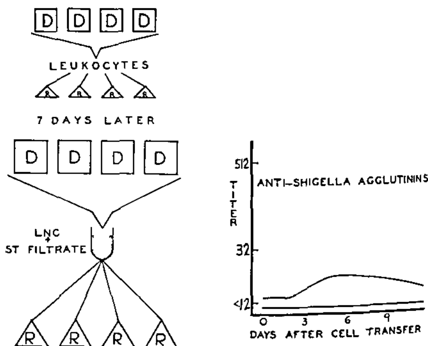


FIGURE 2 Diagrammatic representation of experimental procedure in leukocyte pre injection experiments The prospective donors were bled and the leukocytes were collected and injected intradermally into prospective recipients After a period of 7 days the donors wer sacrificed Lymph node cells obtained from them were incubated *in vitro* with Shigella trypsin (ST) filtrate and transferred to the pre injected recipients In the graph on the right is shown the range of time curves of the recipients

Experiments in the in Vitro Incubation System Involving Various Intervals Between the Pre injection of Donors Leukocytes and the Transfer of Their Lymph Node Cells

Early experiments in this system were carried out with an interval of 6 to 8 days between the pre injection of the donors leukocytes and the lymph node cell transfer [11] When sera of the recipients were examined subsequently no anti Shigella agglutinins could be found at the threshold of measurement In an exploration of the time relations between leukocyte pre injection and lymph node cell transfer it was found that if this

interval was only 1 day the peak titers of the pre injected recipients were indistinguishable from those of the nonpre injected controls At a day interval the geometric mean peak titer was lower and the reduction of the mean titer continued with increasing values of the pre injection interval as can be seen in Figure 3 With increasing values of the pre injection interval it can be seen that up to o days there was essentially

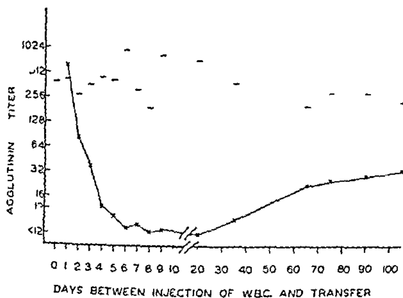


FIGURE 3 Geometric mean peak agglutinin titers of recipient rabbits which had been pre injected with leukocytes of other (donor) rabbits at various intervals prior to the transfer of antigen incubated lymph node cell from the same donor rabbits Geometric mean peak titers of control (nonpre injected) rabbits for each group are hown by the horizontal lines

complete suppression of the effect of the lymph node cell transfer and thereafter a gradual increase of mean titers of recipients up to the longest period tested 107 days at which interval the mean titer was still well below that of the control recipients

Experiments with Lymph Node Cells from Antigen injected Donors

In such experiments recipient rabbits were pre injected intradermally with donor leukocytes at various intervals prior to cell transfer On the day of transfer cells were obtained from lymph nodes regional to injec

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tions of Shigella which had been made 1 2 or 3 days earlier for transfer to the pre injected recipient rabbits

It was found that the results of transfer of lymph node cells from antigen injected donors could also be markedly affected by the pre injection of donor leukocytes at appropriate times The recipients were grouped by donor interval (the interval between injection of antigen into the donor and collection of its lymph node cells) and the pre injection interval (the interval between the pre injection of donor leukocytes and cell transfer) and the geometric mean peak titer of each group was determined In the case of lymph node cells of a 1 day donor interval the curve of mean agglutinin titer versus pre injection interval was similar to that observed in the case of lymph node cells incubated *in vitro* with the antigen Cells obtained at a 2 day donor interval yielded a curve similar in shape to the others but with a difference of 1 day in the pre injection interval required for the reduction of the mean titer to a given level With cells obtained at a 3 day donor interval the curve was again quite similar in shape with a difference of 1 to 2 days from the curve obtained with cells at the 2 day donor interval

The Effect of Variations in the Number of Donor Leukocytes Injected

At a pre injection interval of 6 or 7 days the effect of varying the number of donor leukocytes was studied It was found that the agglutinin titers of the recipients were a function of the number of donor leukocytes pre injected in experiments including intradermal and intravenous injections of the leukocytes The peak agglutinin titers were very low in recipients injected with 10^8 or 10^7 leukocytes as is reflected by the mean

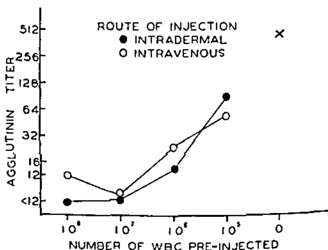


FIGURE 4 Geometric mean peak titers of lymph node cell recipients pre injected with donor leukocytes in various numbers intradermally or intravenously The X (upper right) represents the mean titer for nonpre injected controls

values shown in Figure 4 It can also be seen in the figure that the mean titer was higher when 10^6 leukocytes were injected and still higher following the pre injection of 10^5 leukocytes but even at the last level the mean titer was substantially lower than that of the control recipients which were not pre injected No consistent difference could be found over the range of 10^5 to 10^8 leukocytes between the effectiveness of the intradermal and intravenous routes of injecting donor leukocytes

Pre injection of Leukocytes of Other Species, and of Other Cells of Rabbits

Leukocytes obtained from the blood of chicken cow and horse were injected into recipient rabbits 6 to 7 days before transfer of *in vitro* incubated lymph node cells The geometric mean peak titer of each group was found to be very near that of the nonpre injected control group Recipients pre injected with human leukocytes showed mean titers lower than that of the control group but substantially higher than the groups pre injected with rabbit leukocytes Those pre injected with rabbit erythrocytes had titers similar to that of the nonpre injected controls

In other experiments recipients were pre injected with other cells obtained from rabbits lymph node cells leukocytes of peritoneal exudates and thymus cells It was found that after pre injection with cells from any of these sources the titers were reduced to the same degree as after the pre injection of rabbit blood leukocytes

The Effect of Various Treatments of Leukocytes Prior to Pre injection

Leukocytes obtained from donor rabbits blood were treated in a number of ways and then injected intradermally into recipients The injection of leukocytes treated in a number of these ways was followed by the appearance of anti Shigella agglutinins in titers almost equal to those of the controls indicating that the treated leukocytes had failed to bring about the pre injection effect to a greater or lesser degree Heating the leukocytes at 60° C for 1 hour or treating with iodoacetate at 10^{-} M caused an inactivation of the pre injection effect (mean titers of recipients equal to those of the control animals) Lyophilization or alternate freezing at —70° C and thawing for 3 cycles yielded a slight reduction in mean titer (one half power of) Finally suspension of the leukocytes in distilled water led to a somewhat lower mean titer (1 3 powers of 2 below that of the controls) and a wider range of variations in titer among individual recipients Two forms of treatment did not appear to affect the ability of the leukocytes to induce the pre injection effect, the mean titers of recipients being as low as that of the recipients pre injected with untreated leukocytes x irradiation at 400 r and sonic oscillation In the latter case the activity was found entirely in the sediment after centrifugation

The Effect of Pre-injection of Leukocytes from Individual Donor Rabbits on the Transfer of Lymph Node Cells from Single Donor Rabbits

In all of the experiments described thus far the leukocytes for pre-injection were pooled from the group of rabbits which were to serve as donors for the lymph node cell transfer. Low agglutinin titers of recipients indicating suppression of the transferred cells were similarly observed when the leukocytes for pre-injection were pooled from one group of rabbits and the lymph node cells from another group. In a number of experiments an individual rabbit was used as the source of leukocytes for pre-injection of 2 prospective recipient rabbits and another rabbit was used as the source of lymph node cells for these recipients. Ten pairs of recipients were involved in such experiments and it can be seen in Table I that in the case of 6 of these pairs both recipients showed very low agglutinin titers or none at our threshold of measurement. Of the 20 recipients in such experiments 16 were found to have low titers,

TABLE I. MAXIMUM AGGLUTININ TITERS OF RECIPIENTS PRE-INJECTED WITH LEUKOCYTES OF INDIVIDUAL DONORS AND GIVEN INCUBATED LYMPH NODE CELLS FROM OTHER DONOR RABBITS

Donors of		Peak Agglutinin Titers of Individual Recipients	
Leukocytes for Pre-injection	Lymph Node Cells for Transfer	Pre-injected	Not Pre-injected
A	K	<12 <12	
B	L	<12 <12	384
C	M	32 1024	768
D	N	24 24	1536
E	O	<12 32	1536
F	P	16 24	
G	Q	64 192	
H	R	12 <12	
I	S	<12 384	
J	T	<12 <12	
pool of above	pool of above	<12 <12 <12	
Albino	Chinchilla	<12 <12 24	
		<12 <12	
Albino	Albino	<12 384	128
			512
			512
Chinchilla	Albino	<12 192	
		128 4096	
Chinchilla	Chinchilla	<12 <12	

at or near the limit of detection, the titers of 3 others were in an intermediate range and the titer of 1 was quite within the range of the control (nonpre injected) animals In the lower half of Table I are shown the results of experiments involving albino and chinchilla rabbits As can be seen no consistent difference between rabbits of these two groups was demonstrated

Pre injection of Recipients with Their Own Leukocytes or with Leukocytes of Other Rabbits

Because of the concept that the effect of the pre injection of leukocytes was due to individual specific tissue antigens within the species it was of interest to compare the effect of pre injection of the recipient's own leukocytes with that of leukocytes of other rabbits Such comparisons were carried out the leukocyte suspensions being injected intradermally or intravenously and the results are shown in the upper two rows of Table II It can be seen in the table that the geometric mean titers of the

TABLE II PRE INJECTION OF EACH RECIPIENT WITH ITS OWN LEUKOCYTES OR WHOLE BLOOD OR WITH LEUKOCYTES OF ANOTHER RABBIT

Preparation	Route of Injection	Mean Peak Agglutinin Titers of Recipients Pre injected with					
		Own Leukocytes		Leukocytes of Other Rabbits		Controls (Non pre injected)	
		No of Rabbits	Mean Titer	No of Rabbits	Mean Titer	No of Rabbits	Mean Titer
Leukocyte suspension	i d	18	7 0	17	4 7	11	7 7
Leukocyte suspension	i v	26	7 3	15	4 2	12	7 5
Whole blood	i v	21	8 2	13	5 4	11	7 7

Mean titers expressed as log

groups pre injected with their own leukocytes were in the same range as but somewhat lower than those of the control groups, whereas the groups injected with suspensions of leukocytes from other rabbits showed substantially lower mean titers The finding of a slight reduction in the mean peak titers of rabbits pre injected with their own leukocytes raised the question of whether the manipulation of the leukocytes, in the preparation of the suspensions for pre injection might have caused changes in constituents of the cells which would render them slightly antigenic to their own tissues Accordingly the experiments were repeated with the pre injection of whole blood in an amount containing the same number

of leukocytes The mean titer of recipients pre injected with their own blood was found to be not lower than that of the nonpre injected controls, as shown in the last row of Table II The mean titer of recipients pre injected with blood of other rabbits showed some reduction in titer but not as much as in the case of pre injection with leukocytes

Transfer of the Pre injection Effect by Lymph Node Cells and by Serum

The results of the experiments summarized above indicated that the prior injection of leukocytes could exert a clearly demonstrable effect of suppressing the function of the transferred lymph node cells in the production of the anti Shigella agglutinins A number of observations suggested that this pre injection effect was immunologic in nature the time interval required between leukocyte pre injection and lymph node cell transfer for demonstration of the pre injection effect the ineffectiveness of the pre injection of leukocytes obtained from several other mammalian species and the effectiveness of leukocytes pooled from other rabbits and even sporadically from other individual rabbits but not of leukocytes obtained from the recipient animal itself Because of the likelihood that an immunologic process was involved experiments were undertaken to see whether this effect could be transferred by lymph node cells or by serum

Lymph Node Cell Transfer of the Pre injection Effect

The essential procedure in these experiments was as follows Pooled rabbit leukocytes were injected into the feet of some rabbits and sheep erythrocytes into others After 4 days the animals were sacrificed the popliteal lymph nodes were removed and teased and the cells were washed and transferred to irradiated recipients (50×10^6 cells per recipient) The rabbits from which the blood leukocytes had been obtained for foot pad injection were then sacrificed their popliteal lymph nodes were excised and teased and cells obtained from these were incubated with ST filtrate and then transferred to the same recipients 2 hours after the first transfer This interval had been reduced to hours in order to avoid effects of active immunization of the recipient by the lymph node cells of the first transfer (The plan of these experiments is shown schematically in Figure 5)

The results obtained in such experiments are summarized in Table III as the geometric means and distributions of maximal agglutinin titers of recipients It can be seen in the table that the mean and distribution of peak titers of recipients given prior lymph node cell transfers from leukocyte injected rabbits differed substantially from those of the con

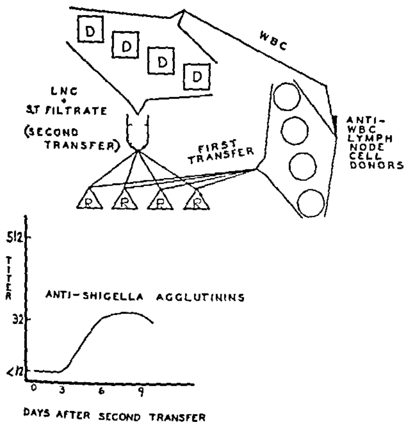


FIGURE 5 Schema of lymph node cell transfer of the pre injection effect Donor rabbits (D) were bled and the leukocytes were injected into the foot pad of intermediate donors (O) After 4 days the latter rabbits were sacrificed and cells obtained from their popliteal lymph nodes were transferred to the recipient rabbits Two hours later the same recipients were injected with antigen incubated lymph node cells obtained from the original donors A typical antibody time curve found in recipients of the two cell transfers is shown below the schema

trol recipients which were not given the first antileukocyte cell transfer The two other control groups – those given a first cell transfer from sheep erythrocyte-injected donors and those given heated antileukocyte lymph node cells – showed means and distributions of titers quite similar to th control recipients which had not had the first cell transfer at all A transfer of the pre injection effect by lymph node cells was thus quite clearly demonstrated

Transfer of the Pre injection Effect by Serum

The plan of the first group of such experiments shown schematically in Figure 6 was as follows 4 to 6 adult rabbits were bled and 10–30 × 10⁶

TABLE III Transfer of Pre injection Effect by Lymph Node Cells from Rabbits Injected with Rabbit Leukocytes

Material Injected into Donors of First Cell Transfer	Anti Shigella Agglutinin Titers of Recipients After Second Cell Transfer				
	Number of Recipients	Mean Titer (log)	Percentage Frequency of Ranges of		
			128 or higher	24–96	<12 16
Rabbit leukocytes	65	5 5	25	34	41
Rabbit leukocytes (heated lymph node cells transferred)	14	8 8	93	7	0
Sheep erythrocytes	28	8 1	86	14	0
Controls (no first cell transfer)	35	8 2	85	15	0

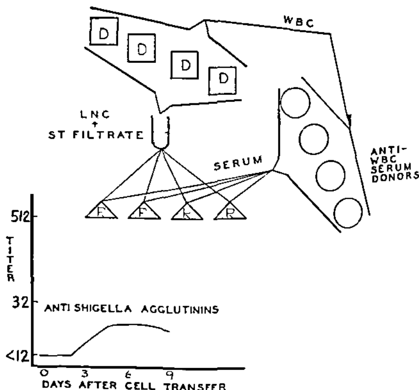


Figure 6 Schema of passive transfer of the pre injection effect by anti rabbit leukocyte serum A number of adult rabbits (D) were bled and leukocytes were collected for injections into other adult rabbits (O) which were bled 8 10 and 11 days later On the day of cell transfer the original donor rabbits were sacrificed and their lymph node cells were incubated *in vitro* first with Shigella trypsin filtrate then with the anti rabbit leukocyte serum before transfer to the recipients The average curve of recipient antibody titer *versus* time after cell transfer is shown below the schema

of the leukocytes thus obtained were injected intradermally into 4 to 8 other adult rabbits The latter were bled usually after 6 days and the pooled serum was injected intravenously in amounts of 10 to 0 ml into the prospective recipients The donors of the leukocytes were sacrificed and their lymph node cells were incubated *in vitro* with ST filtrate and transferred to the same recipients - to 3 hours after the serum injection When the sera of the recipients were subsequently tested for anti Shigella agglutinins it was found that some recipients failed to develop agglutinins or did so to very low titer In almost every experiment there were however recipients which developed higher titers sometimes as high as those of the controls It can be seen in the first row of Table IV

TABLE IV Effect of Anti Rabbit Leukocyte Serum on Agglutinin Titers of Recipients of In Vitro-Incubated Lymph Node Cells

Anti Leukocyte Serum Pool	Number of Rabbits Used for Leukocyte Pool	Mode of Injection of 10 ml Serum	Anti Shigella Agglutinin Titers of Recipients				
			Number of of Recipients	Mean Titer ($\log_2$)	Percentage Frequency in Ranges of		
					128 or higher	24–96	<12
Early serum pools (13)	4–6	2 hours before cell transfer	62	5 0	19	36	45
Pool C	4–6	2 hours before cell transfer	12	5 2	25	42	33
Pool 10–11	60	2 hours before cell transfer	27	4 4	0	59	41
Pool 10–11	60	Incubated with lymph node cells	24	3 6	0	25	75
Pool 12 18	60	Incubated with lymph node cells	17	3 5	0	18	82
Controls (No serum)			87	8 8	93	7	0

that although the mean and distribution of peak agglutinin titers were substantially different from those of the control recipients 19 per cent of these serum injected recipients developed titers in the range of the controls and another 36 per cent had titers in the middle range A pool of such serum pools (pool C) yielded similar results

It was considered likely that the inconstancy of the suppressive effect was due to the fact that the serum pools had been obtained by the injection of leukocytes pooled from too small a number of rabbits (4 to 6) to insure an adequate sampling of the tissue antigens randomly distributed among the heterogenous population of rabbits Accordingly leukocytes were pooled from 60 rabbits for injection into 8 to 10 other rabbits Sera obtained from these rabbits 8 10 and 11 days after injection of the leuko

cytes were pooled and 10 ml quantities were injected intravenously into recipient rabbits 2 to 3 hours before transfer of antigen incubated lymph node cells which had been obtained from rabbits other than those used for obtaining the leukocytes It was found that these recipients (Table IV, third row) showed a lower mean titer of anti Shigella agglutinins and a substantially different distribution than those given the sera obtained with leukocytes from fewer rabbits However, even with the use of these sera it can be seen that the agglutinin titers of more than half of the recipients were in the middle range Also a considerable range of titers was found on occasion among recipients given 10 ml of the serum before cell transfer Because of the possibility that the failure of an antileukocyte serum to suppress the cell transfer effect in the case of a particular recipient might be due to competition for certain antibodies between antigens abundant in the tissues of the recipient host and the same antigens in the transferred lymph node cells the experimental procedure was altered to one which would afford the lymph node cells rather than the host tissues prior contact with the antibodies in the serum Thus 10 ml of the anti leukocyte serum were incubated with the lymph node cells to be transferred in a roller tube apparatus for 30 minutes at 37° C and the entire suspension was then injected intravenously into the recipient rabbit The first few experiments by this procedure were done with antileukocyte serum pools 10 and 11 and the data obtained in these experiments are summarized in the fourth row of Table IV A comparison with the row above (the same serum pool injected before the cell transfer) shows a reduction in geometric mean titer by almost a power of 2, and a reduction of the percentage of titers in the middle range from 59 to 25 Other anti leukocyte serum pools obtained and tested by these procedures yielded similar results (Table IV fifth row)

Evidence that the agent in the serum which produced the pre injection effect was an antibody was obtained in experiments of several types First, several pools of sera from presumably normal rabbits were tested at 10 ml per recipient without substantial effect on the subsequent agglutinin titers These included some sera from pre-injection bleedings of animals used for production of antileukocyte serum pools Second portions of antileukocyte serum pools were absorbed with mesenteric lymph node cells of rabbits by suspending such cells in the serum incubating at 37 C, then at 4 C for a day removing the cells and repeating the process once more Such absorbed sera were used in cell transfer experiments in each case in direct comparison with the original serum used in the same volume The mean agglutinin titers of recipients of cells treated with such serum were found to be substantially higher than those for which the original serum was used in all such experiments often being as high as those of the control (nonserum injected) recipients Third heating of

antileukocyte serum at 56° C. for 30 minutes did not alter its effect on subsequent agglutinin titers of cell transfer recipients Finally globulin was prepared from several antileukocyte serum pools by adding ammonium sulfate to half saturation or phosphates to 1 33 M The precipitates thus obtained were dissolved and diluted to the original volume of serum and used similarly in cell transfer experiments The agglutinin titers of recipients of cells treated with either preparation of globulin were usually at or below the threshold of measurement

The effect of anti rabbit leukocyte serum on lymph node cell transfer was also studied in the case of individual donors In such experiments an individual rabbit scheduled to be a donor of lymph node cells was bled and the leukocytes were collected washed and injected into another rabbit The latter was bled prior to the injection and 8 and 10 days later On the day of cell transfer the donor rabbit was sacrificed and the cells obtained from its popliteal lymph nodes were incubated *in vitro* with ST filtrate One portion of the washed cell suspension was incubated with the serum of the rabbit obtained before it was injected with the donor s leukocytes and another portion or portions with the serum obtained after the leukocyte injection Each mixture was transferred to an individual irradiated recipient A total of o pairs of sera were tested in 47 recipients (o of the preliminary sera and 7 of post injection sera) Among the recipients of lymph node cells incubated with serum obtained before the leukocyte injection it was found rather unexpectedly that one half showed agglutinin titers below the range of nonserum injected controls 3 of the o having very low titers On comparing anti Shigella titers of recipients given pre and post injection sera respectively of each leukocyte injected rabbit minor differences were found in a number of pairs in either direction All of the greater ratios in titers (sixfold to thirty two fold) were in the direction of lower titers in recipients given antileukocyte serum than in those given serum obtained from the same rabbit before the injection of leukocytes Such differences were found in 9 recipients involving 8 serum donors or half of the 16 leukocyte injected rabbits whose preliminary serum led to titers high enough for the demonstration of differences of this magnitude

Comments

The Immunologic Basis of the Pre injection Effect

The experimental data summarized above indicate clearly that the production of an antibody (agglutinins to Shigella) in recipients of transferred lymph node cells can be markedly suppressed by the effects of a prior injection into the recipient s tissues of homologous leukocytes As was indicated above several observations on the pre injection effect sug

gested that this was immunologic in nature The finding that this effect could be transferred by lymph node cells or by serum of leukocyte injected rabbits further supports this concept

The antigens involved in the pre injection effect are presumably individual specific tissue antigens of rabbit leukocytes The random distribution of these antigens is the basis for a number of the observations made in this study the effectiveness in pre injection of leukocytes pooled from rabbits other than the donors of lymph node cells, the range of pre injected recipients agglutinin titers (and hence of degrees of suppression of the transferred cells) found in individual donor experiments (Table I), and the similar range in the individual donor experiments involving passive transfer by serum

The data in Table I indicate the complexity of the problem and imply the presence of several antigens in rabbit leukocytes The necessary conditions for the suppression of the transferred cells (low agglutinin titers) include the existence of at least one antigen common to the tissues of the donor of the leukocytes and the donor of the lymph node cells, and the absence of those antigens in the tissues of the recipient so that it can react immunologically to them Intermediate recipient agglutinin titers indicating partial suppression of the transferred cells, could reflect quantitative relations among the antigens or relative degrees of antigenicity of the tissue factors perhaps on the basis of degrees of difference in configuration between antigenic groupings of the tissue factors involved Thus it is possible to encounter any combination of results in experiments of this kind carried out in a genetically heterogeneous population The fact that of 20 recipients shown in Table I as receiving the pre injected leukocytes and the transferred lymph node cells from two different individual rabbits 16 had markedly reduced titers – that is the fact that the probability of the occurrence of a common antigen in the leukocytes of two rabbits chosen at random is high – suggests that there are several individual tissue antigens in leukocytes of rabbits and that each rabbit carries a few of these with a considerable degree of overlapping The frequency of occurrence of low titers in these recipients also suggests that each rabbit carries at most half of the total number of such antigens since the probability was so high that a randomly chosen recipient would not carry the antigens common to the two randomly chosen donors It is of interest that Medawar [14] in his study of the immune response to rabbit skin homografts (which can also be elicited by the injection of rabbit leukocytes [14]) obtained evidence of at least seven antigens in rabbit skin In the individual donor experiments by passive transfer the frequency with which suppression of the transferred lymph node cells was demonstrated and the wide range of degrees of such suppression encountered are also consistent with this interpretation

regarding the number and distribution of rabbit leukocyte antigens The data of the individual donor passive transfer experiments also indicate that rabbits can carry serum antibodies to some of these tissue antigens as in the case of the ABO system of human erythrocyte antigens

The Leukocyte Pre injection Effect in Relation to Tissue Homotransplantation

The transfer of lymph node cells may be considered as a particular case of tissue homotransplantation One of the most thoroughly studied areas of tissue transplantation is that of skin homografting and the pre injection effect in the lymph node cell transfer system is analogous in some respects to the accelerated rejection of a second skin graft the second set phenomenon described by Medawar in the case of a second skin graft from a given donor to a given recipient [13] Observations have in fact been made in these studies which are parallel to those reported in skin homograft studies with respect to effect of time and dose of the first graft [2, 12] ineffectiveness of erythrocytes for the primary contact between donors and recipient s tissues [15] and the effects of a number of treatments on the efficacy of the first tissue transferred [4, 16] Also the property of accelerated rejection of skin homografts and of resistance to transplanted tumor has been transferred by lymph node cells by workers in these fields [2, 17] and such transfer of the pre injection effect has been demonstrated here in the lymph node cell transfer system

With respect to transfer by serum, the data summarized above indicate clearly that the effect on lymph node cell transfer which is actively induced in recipient animals by the pre injection of rabbit leukocytes can also be passively transferred by anti rabbit leukocyte serum very probably by an antibody In the fields of tissue transplantation mentioned above it has not been found possible in most of the studies reported to demonstrate passive transfer of transplantation transfer immunity by serum [2, 3, 17] although some reports suggestive of such transfer have appeared [1, 20, 21] The difference between the effectiveness of serum transferred transplantation immunity in the case of transferred lymph node cells and of the tissues referred to respectively, may reflect a difference in mechanism of immunity More likely, it may reflect a difference of accessibility first of the antigens of transferred cells to the antibody forming apparatus of the recipient and second of serum contained antibodies in the recipient to the transferred cells

Implication of These Data as to the Function of the Transferred Lymph Node Cells

It is clear from the data presented above that in this experimental situation the pre injection of homologous leukocytes or the transfer of lymph

node cells or serum from leukocyte injected rabbits has caused an alteration in the host tissue environment into which the lymph node cells are transferred this alteration being specific with respect to the transferred cells The fact that this alteration resulted in the failure of appearance of agglutinin which normally follows the transfer of Shigella antigen incubated lymph node cells supports the thesis that some function of transferred lymph node cells is involved in the production of the antibody found under these circumstances

This thesis is further supported by the data obtained with the transfer of cells from antigen injected donor rabbits Following the transfer of such lymph node cells agglutinins have been found to appear in the recipient's serum after a variable number of days, this interval depending on the number of days allowed between the injection of antigen into the donor and the transfer of its lymph node cells In the case of lymph node cells obtained at each of the intervals tested transfer into a pre injected recipient resulted in failure of agglutinins to appear Since the transfer of lymph node cells into a specifically adverse environment even within one day of the time antibody would have appeared in the recipient resulted in the failure of appearance of that antibody, it seems likely that the function of the transferred cells is not merely to contribute some factor which the tissues of the recipient can use to undertake the synthesis of the antibody, but rather to carry out at least a major part of the synthesis of the antibody themselves

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GENERAL DISCUSSION

CHAIRMAN STETSON I should like to thank the speakers at this session for their cooperation It would be unthinkable after the interesting topics discussed by Dr Brent and Dr Harris not to have some general discussion I know Dr Brent is anxious to make a few remarks so I will call on him to do so at this time

DR BRENT Since there is so little time for general discussion perhaps I had better not take up more time with further comments

RUDOLF E WILHELM (Detroit, Michigan) Dr Brent have you ever observed necrotic reactions when the immune lymph node cells were injected into the skin of the donor?

DR BRENT Yes when the reaction is a particularly strong one (and the strength of reaction does vary somewhat from animal to animal) we have had slight necrosis usually toward the center of the reaction site In the *rabbit*, necrosis is more extensive In the three rabbits tested with immune lymph node cells necrosis of host tissue always occurred

CHAIRMAN STETSON I should like to speak for a few seconds to Dr Harris's remarks about the antigenic overlap in experimental animals particularly in hybrid rabbits In our model immunization with Freund's adjuvant against spleen cells of one rabbit produces white graft immunity to subsequent homografts from the same rabbit and sometimes accelerated rejection of homografts from any other rabbit Sometimes there is more and sometimes less of this but we have had reason to think this is another expression of this antigenic overlap that Dr Harris brought up

DR BRENT Perhaps I could comment here Billingham and I have tried to transfer immunity passively with immune mouse sera but we have consistently failed to do so Our experiments of course differed from those of Dr Stetson in that we did not use Freund's adjuvant in the immunization of the serum donors

We did however use hyperimmune sera — the serum donors having been first immunized with homologous skin grafts and subsequently with several intraperitoneal injections of tissue cell suspensions Neither the injection of very large nor of very small doses of immune serum has ever transferred transplantation immunity either in normal or immunologically tolerant mice

It would certainly be of the greatest interest to find out whether Dr Stetson and his colleagues can transfer immunity to tolerant animals

If immunity is successfully transferred one would expect a skin graft on a tolerant mouse to be destroyed by the injection of immune serum from a specifically immunized animal

CHAIRMAN STETSON We quite agree Dr Brent and we will look forward very much to an opportunity to do that experiment

MERRILL W CHASE (New York New York) I don t know whether immunology is in crisis, perhaps immunologists are! Surely all of us are in favor of antibodies! In dealing with questions that are only partly opened up decisions are not to be reached easily – for example the question as to how antibodies are produced or the sequence of events that occur and lead to stimulation of the immunological apparatus

It would seem that Dr Brent has at hand a powerful tool to use in investigating the problem of tissue antigens in quite a new way With it perhaps one could ascertain whether the antigens that are involved in homograft rejection are actually as labile as has been believed Will his reactions be secured after the cells have been frozen? What if he tested the supernatant fluids alone after removal of the cells? Very many elegant experiments should be possible with this method

With regard to Dr Stetson s experiments and his interpretation it would seem that a positive finding with use of cytotoxic antibodies does not decide the issue of mechanism Could there not exist two separately effective pathways for tissue rejection circulating antibodies on the one hand and cellular elements on the other either one potentially capable of causing tissue rejection? The problem would seem to be to learn by direct observation which pathway is utilized by the animal that rejects tissue For example a biochemist with use of a liver ' brei may find certain metabolic pathways to operate with respect to a particular substrate but when the composition of afferent and efferent blood of the whole organ is studied it may turn out that the body actually utilizes a different pathway than had been inferred from study of the total enzymic potentialities

If specific cytotoxic antibodies can be secured in adequate concentration even artificially concentrated over normal levels should they not be expected to cause tissue rejection when administered? But for interpretation any such finding would require balancing against the concentrations of cytotoxic antibodies as they usually occur in animals that reject homografts and against possible alternate pathways of rejection

GUY A VOISIN (Paris France) I have listened with great interest and pleasure to the three fascinating papers given during this session Dr Stetson s and Dr Harris s papers bring new evidence favoring the role of antibodies in the rejection of homografts and homotransplanted cells

as we suggested from our experiments Dr Stetson made clear that iso-antisera alone are often able to passively immunize a recipient toward a homograft As for the question of 'overlapping antigens presented by Dr Harris, I agree with such a possibility whatever its precise meaning may be In experiments which are still in progress in my laboratory it has been noticed that rabbits injected with living blood cells from twenty different donor rabbits were able to reject in an accelerated way a skin graft coming from a twenty first nonrelated rabbit Concerning Dr Brent's extremely interesting presentation I have to point out a technical difficulty which often arises when injecting cells intracutaneously that is the possibility of nonspecific inflammatory reactions closely resembling delayed hypersensitivity skin reactions We have been faced with that difficulty in studying delayed hypersensitivity to tuberculin Nevertheless it may well be possible that with an appropriate number of cells the specific reaction will not be overshadowed by the nonspecific one That seems to be the case in Dr Brent's work

X

Hormones and Allergic Responses

Chairman BRAM ROSE M D (*Montreal, Canada*)

38

Hormones and Allergic Responses

BRAM ROSE M D

(Montreal Canada)

Pituitary Adrenal Hormones

The exact mechanism by which the hormones of the anterior pituitary and the adrenal cortex exert profound effects on some allergic responses in animals and man remains unknown in spite of much investigation By way of introducing this section of the symposium it seems pertinent to enumerate those hormones which are capable of exerting an effect on various mechanisms involved in the immune reaction Beginning with the anterior pituitary two complexes are of importance These are first adrenocorticotrophic hormone (ACTH) and second somatotrophic hormone (STH) ACTH acts via the adrenal cortex to stimulate the release of a variety of steroid complexes which in turn produce a multiplicity of effects ACTH has no direct activity of its own and is devoid of activity in the absence of the adrenals STH on the other hand apparently acts directly and is independent of the state of the adrenal It possesses both inflammatory and anticortisone properties

Turning now to the adrenal cortex the most important steroid complex liberated in man relative to immune mechanisms is cortisol or hydrocortisone (17 hydroxycorticosterone)[15] It is important to recognize that in some animal species, such as the rat, mouse and rabbit the major steroid released appears to be corticosterone This may be of considerable significance in the interpretation of results obtained following the administration of ACTH on the one hand and cortisone on the other to various animal species Some of these will be discussed by Dr Darrach (Chapter 39) It is these three hormones ACTH STH and cortisol with which we are chiefly concerned Neither the electrolyte and water controlling hormones such as desoxycorticosterone or aldosterone nor the androgens such as testosterone which are also elaborated by the adrenal cortex appear to exert any known major effect on allergic mechanisms or immune responses

In keeping with the general thesis that the adrenal is related to resistance in a general and nonspecific manner it has been thought that this holds as well for immune responses The problem cannot be resolved as simply

as this would seem to imply, particularly in view of the specific mechanisms involved some of which will now be considered

Anaphylaxis, Local and General Allergic Phenomena

Effect of Adrenalectomy As far back as 1926[3] it was observed that death from anaphylaxis was more readily induced following extirpation of the adrenals in the dog Similar observations have been reported from time to time and include the guinea pig[5] rat[10] mouse[99] and rabbit[11] In surveying these findings it would appear that the results are more convincing in the rat and mouse than the guinea pig and rabbit Both the Arthus phenomenon and the lesions of necrotizing arteritis induced in the rabbit by the administration of foreign serum occur more readily and with greater severity when the adrenals have been ablated[11 15]

Effect of Steroid Administration It is apparent that the administration of ACTH or cortisone to sensitized rats and mice will greatly suppress generalized anaphylaxis[15 71 91] The administration of these hormones to actively sensitized rabbits suppresses the appearance of the Arthus phenomenon as well as the lesions of delayed hypersensitivity such as vasculitis carditis and glomerulonephritis subsequent to an injection of a large dose of foreign protein[4 1 22 32 64]

In the guinea pig on the other hand anaphylactic shock is uninfluenced by the administration of ACTH or cortisone[27 58 63] The reasons for this are not clear although it may relate to the fact that histamine is the predominant metabolite released during anaphylaxis in this species[10] or that relatively small quantities of antibody are involved A localized form of guinea pig asthma induced by subjecting sensitized guinea pigs to an atmosphere of aerolosized antigen is suppressed if the animals are first treated with cortisone or ACTH[21] This type of bronchospasm however can be inhibited as well by various nonspecific substances such as sodium nucleate Furthermore if bronchospasm is induced by aerosolized histamine in guinea pigs neither ACTH nor cortisone exerts any obvious protection[65]

Other allergic responses which do not seem to depend on histamine release are also amenable to the effects of the pituitary adrenal hormones These include granuloma formation in the guinea pig induced by the administration of egg albumen in adjuvants[23] experimental encephalomyelitis[49] and the tuberculin reaction in the skin[18] Passively transferred delayed hypersensitivity to 2 4 dinitrochlorobenzene could be sharply reduced in guinea pigs treated with ACTH but not with cortisone[88]

In man both the immediate direct skin test and the passive transfer (Prausnitz Kustner) test which depend on histamine release are uninfluenced by the administration of ACTH, cortisone or its derivatives[25 60]

It is of some interest therefore that the lesions of hypersensitivity to cold which consist of urticaria and angioedema and which are also dependent upon the release of histamine are refractory to the administration of the steroid hormones[81] Most other allergic states such as allergic rhinitis (hay fever) asthma atopic dermatitis contact dermatitis and certain forms of urticaria respond clinically to ACTH or cortisone compounds[2, 7, 8, 72] Although it is probable that histamine release plays some role in these conditions it is very likely that some other mechanism is responsible otherwise one might expect to find the antihistamines more effective Furthermore there is no clear correlation between the changes in histamine metabolism and appearance or disappearance of symptoms[6]

Antibody Formation

The status of adrenocortical hormones relative to antibody formation is anything but clear despite a considerable quantity of investigation There was no general agreement regarding the effects of adrenalectomy in the early literature Apart from such variables as species difference and the antigen antibody system used failure to confirm the completeness of adrenalectomy by autopsy in order to rule out the presence of accessory or remnant tissue contributed to the difficulties in the interpretation of results Furthermore the potency of early extracts as ACE (adrenocortical extract) while adequate to maintain adrenalectomized animals was not sufficient to exert measurable effects on antibody

Effects of Adrenalectomy In 1918 Gates[29] was unable to demonstrate any change in antibody to hens RBC or typhoid bacilli in partially adrenalectomized guinea pigs Using rats and rabbits Marine[48, 94] found an increase in both hemolysins and agglutinins following adrenalectomy However the first clear demonstration of an increase in precipitin to horse serum in rabbits by means of adrenalectomy was that of Murphy and Sturm,[70] and their observations have since been repeatedly confirmed[11, 15]

Effect of Pituitary Adrenal Hormones The effects of adrenocortical steroids on antibody stems from the early work of Fox and Whitehead[28] who concluded that antibody formation was enhanced The extracts at their disposal were crude and poorly standardized Nevertheless similar findings were reported by Dougherty and White[1] and partially confirmed by Hammond and Novak,[41] who found that the hemolysin and agglutinin titer to sheep RBC were enhanced when an anamnestic response was induced in sensitized rabbits by the administration of a lipo-adrenal extract In keeping somewhat with this general idea Halpern *et al*[9] showed that if the antibody level in sensitized rabbits is first depressed by the administration of a neutralizing dose of antigen the subsequent

reappearance of antibody in the serum for the first 48 hours is enhanced if the animals are treated with cortisone However after this initial period there is a definite lag in the titer as compared with control animals The demonstration of an anamnestic rise in antibody following the administration of ACE to sensitized animals could not be confirmed by Fischel *et al*[21] as well as Eisen *et al*[20]

In contrast to these findings and more in keeping with the effects of adrenalectomy Germuth Ottinger and Oyama[5] observed that administration of ACTH or cortisone to ovalbumin sensitized rabbits markedly suppressed antibody levels and this has been confirmed many times not only in rabbits[5 12 2 31] but also in guinea pigs and mice[15 3] Similar findings have been reported for different systems such as a decrease in antibody titers to para B vaccine in rabbits[26] as well as neutralizing antibody and hemagglutinin inhibiting antibody to viruses[55]

As to the mechanism by which such changes are produced there is again no unanimous opinion Fischel *et al*[25] sensitized rabbits both by active and passive means They noted that whereas antibody suppression occurred during active sensitization no effect was observed on the antibody titers of the passively sensitized animals as a result of the administration of ACTH or cortisone It was inferred that the effect of the hormone must be directed to the site of antibody production since if it were due to a protein catabolic mechanism the passively transferred antibody should also have been altered Using *Pasteurella pestis* as antigen in three month old rats Hayshida and Li[46] studied the effects of ACTH and STH (somatotrophic hormone) on antibody formation They noted that when ACTH was given in a dose sufficient to suppress growth antibody formation was also suppressed When STH was given alone growth was enhanced but antibody production was not influenced The administration of STH along with ACTH resulted in normal growth and antibody production They conclude therefore that the mechanism of antibody suppression by ACTH is mediated by its protein catabolic activity Similar results using ACTH STH and cortisone were reported by Cordon and Benditt[34] Stimulation of both antibody production and plasma cell production by the administration of STH to rats was observed by Schelin *et al*[88]

Consistently negative results have been obtained in man Thus De Vries[14] was unable to modify circulating antibody levels by the administration of ACTH Neither could Mirick[66] influence mouse protective antibody nor Larson and Tomlinson[56] inhibit precipitin formation to pneumococcus polysaccharide by the administration of ACTH and cortisone These hormones were also incapable of reducing the formation of diphtheria antitoxin when toxoid was administered to patients by Havens *et al*[45] It is also apparent that skin sensitizing antibody is not

affected since skin tests persist in serum sickness in man even though symptoms may be controlled[9, 2, 6, 93]

In spite of these controversial results there appears to be a certain trend of events which point to differences in the type of antigen used and the species involved Thus Hanan and Overman[4] and Hanan and Oyama[4] feel that the size of the antigen particle may be of importance For example somewhat consistent results have been obtained with the steroids when small soluble antigens such as the serum proteins are used whereas when sheep red cell stroma which is large and particulate is employed a much smaller effect is noted Possibly there are several sites or elements of the reticuloendothelial system which capture antigen some of which are sensitive to steroids and others of which are not However Downs and Whitmire[18] were unable to confirm the difference in antibody response to small and large antigen The administration of cortisone had no effect on complement fixation titers of rabbits immunized with either whole or sonic treated rickettsial antigens

It is improbable that the steroids exert their effect by inducing lysis of the lymphocytes with release of gamma globulin for if this were the case one would expect to find an increase both in antibody and gamma globulin Such is not the case in the majority of experimental procedures Thus adrenalectomy is followed by a marked increase in the size of the lymph nodes and thymus as well as increased lymphoid activity of the spleen[70] At the same time there usually occurs an increase in the gamma-globulin of the serum in immunized animals The reverse is also observed for when cortisone is administered to such animals a lymphopenia melting away of lymphoid tissue and a decrease in the gamma globulin can be noted[12] Furthermore in those clinical states such as penicillin reactions polyarteritis and disseminated lupus erythematosus in which an antigen antibody mechanism is assumed and in which there is usually an increase of the gamma globulin suppression of the symptoms is accompanied by a lowering of the gamma globulin as a result of the administration of the steroid hormones[76, 81] A more plausible explanation would seem to be that suppression of the lymphoid mass reduces or cuts off the supply of the antibody precursor to the plasma cell or perhaps the precursor of the plasma cell itself as is believed by some This is further substantiated by the effects of irradiation and nitrogen mustard on antibody formation Thus both are capable of suppressing the formation of antibody if administered prior to the sensitizing dose[7, 16] However the transfer of lymphoid cells from a sensitized rabbit prior to the appearance of antibody to an irradiated animal is followed by a heightened antibody response[44] and it would therefore appear that the precursor of antibody is sufficient to continue the process if plasma cells are available

Eosinophils, Mast Cells, Histamine, and Serotonin It is of considerable interest that eosinophils mast cells histamine and serotonin (5 hydroxy tryptamine) all of which are intimately related to anaphylaxis are also influenced by the hormones of the anterior pituitary and the adrenal cortex The eosinophil has been regarded as a hallmark of allergy for many years since the incidence of eosinophilia in almost all allergic states of man with few exceptions is very high [77] Hypersensitivity in animals is also associated with both local and general eosinophilia [85] As pointed out by Speirs [9] in his excellent review eosinophilia can be induced readily in the mouse by the administration of pollen extracts as well as a number of antigenic and other substances Apparently it is not the antigen per se which induces the eosinophilia but the reaction of the sensitized organism to the specific antigen which is responsible Thus following sensitization subsequent injections of antigen will induce intense eosinophilia

Removal of the adrenals will enhance the increase in eosinophils whereas the administration of ACTH or cortisone will virtually abolish it [92] Samter *et al* [86] were able to induce eosinophilia in normal guinea pigs by a factor obtained from the lungs of guinea pigs dying of anaphylactic shock It is possible that this could be due to the antigen responsible for inducing anaphylaxis part of which could have remained unaltered However this is unlikely According to Muehrcke [60] and Godlowski [33] adrenocortical hormones have a direct lytic effect on eosinophils In his experiments however Speirs [9] found that the eosinophils decreased first in the blood and later disappeared from the peritoneal cavity regardless of whether the steroid was administered by injection directly into the peritoneal cavity or by other routes

In man hypofunction of the anterior pituitary or the adrenal cortex is occasionally accompanied by a moderate eosinophilia However the incidence of allergy in such patients is apparently no greater than in the normal individual The well known reduction in eosinophils following the administration of ACTH to man as shown originally by Hills *et al* [47] has been amply confirmed however the mode of action is still unknown

Two of the chief metabolites associated with antigen antibody mechanisms leading to anaphylaxis in the experimental animal are histamine [18] and serotonin (5 hydroxytryptamine) [98] One of the major sources of both is the mast cell [100] The administration of cortisone induces a considerable reduction in mast cell population or indeed a virtual disappearance in rather rapid time of mastocytomas in the dog [6] An increase in the histamine content of the tissues of the rat [64, 8] and rabbit occurs [101] following removal of the adrenals At the same time the animals become more sensitive to anaphylaxis and the effects of histamine [78] The converse was not demonstrated until more recently when more potent steroids of the adrenal cortex were made available Even then it was not possible to show

a direct effect on histamine until histamine liberators were employed Apparently the administration of steroids to intact animals has no demonstrable effect in that the tissue histamine remains at normal levels If however the tissues are first depleted of their histamine content by the administration of a potent histamine liberator such as Tween or 48/80 the reaccumulation of histamine which would normally occur on withdrawal of the histamine liberator can be prevented by the administration of cortisone as shown first by Goth *et al*[2] and later by Halpern and Briot[29] The mechanism by which cortisone prevents the reaccumulation of histamine is clarified by the observations of Schayer *et al* who used C^{14} labeled *l* histidine When this is administered to normal rats histidine is converted to C^{14} labeled histamine presumably by the action of the enzyme histidine decarboxylase Administration of cortisone prevented the conversion of histidine to histamine so labeled There are two possible explanations It is known that adrenalectomy decreases the activity of the enzyme histaminase as shown by Karady *et al*[51] and later confirmed by Kahlson *et al*[30] Possibly therefore this enzyme is potentiated by cortisone and may account for the increased destruction of histamine There is some supportive evidence for this in the pregnant woman in whom both an increase of histaminase activity of plasma[1] and increased excretion of glucocorticoid activity in the urine occur[6] The second possibility is that histidine decarboxylase activity may be inhibited There is evidence that the administration of ACTH and cortisone induces an increased histamine output in the urine[28, 6, 84] as well as its precursor histidine[8] In effect these findings are somewhat difficult to correlate since depletion of histidine should decrease the quantity of histamine available for excretion by the kidney In contrast to these findings are those reported by Duner and Pernow[19] It was noted that the urine histamine content was considerably elevated in a patient with Loeffler's syndrome The administration of ACTH caused a fall in the urine histamine to normal levels and a decrease in eosinophils Withdrawal of ACTH was followed by a return of both histaminuria and eosinophilia but there was no direct relation between the blood eosinophils and urine histamine

Attempts to show a direct action of steroids on the effects of histamine *in vitro* or on the histamine induced wheal and flare in the skin of man have been unsuccessful However Lefcoe[57] has recently demonstrated that the addition of hydrocortisone hemisuccinate to a bath in which tracheal rings are suspended will suppress the contraction of smooth muscle usually obtained on the addition of histamine or acetylcholine A relaxation of smooth muscle was also observed

Summary While it is abundantly clear that many of the basic mechanisms of the immune response in animals and man are profoundly altered

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Summary While it is abundantly clear that many of the basic mechanisms of the immune response in animals and man are profoundly altered

by the hormones of the anterior pituitary and the adrenal cortex the actual means by which these occur remains obscure Attempts to correlate adrenocortical activity in patients with various forms of allergy have been controversial Thus Rose Tyles and Venning[83] were able to show a tendency to decreased activity in older patients with the more chronic forms of allergy However similar changes were observed in patients with rheumatoid arthritis[9] and an actual increase in the blood 17 hydroxysteroids of children with acute symptoms was noted by Siegel *et al*[90]

It seems fair to say that in animal species the type of antigenic stimulus and the time of administration of these hormones are of considerable importance In general reactions of the delayed variety, such as the tuberculin reaction in the skin granuloma formation as well as the Arthus reaction, vasculitis carditis glomerulonephritis and the like, are amenable to control being exacerbated by adrenalectomy or suppressed in part by the administration of these hormones Their effects in acute manifestations such as anaphylaxis depend in part on the nature of the metabolite released for in those animal species in which histamine is the major factor in the production of symptoms they are without effect This also holds for other histamine mechanisms such as the immediate skin test in man or histamine administration to the guinea pig The symptoms of anaphylaxis in the mouse and rabbit are apparently due in the main to serotonin and in these species it is apparent that the steroid hormones are effective Other basic mechanisms such as antibody formation lymphocytes and other cells involved are also susceptible to the action of these steroids It is unlikely under the circumstances then that the results obtained in the allergic states in animals and man are simply due to the anti inflammatory action of these compounds as suggested recently by Germuth[30]

Thyroid Hormone

In general the relation of thyroid activity to hypersensitivity or allergy is opposite to that of the pituitary adrenal hormones For example thyroidectomy decreases both the tuberculin reaction and anaphylaxis[55] [Leger et al [9] noted that resistance to the egg white reaction in rats was increased by removal of the thyroid Conversely all of these reactions were made much more severe by the administration of thyroxin An interesting finding was reported by Gotzl and Dragstedt[36] who noted that removal of the thyroid leads to a decrease in the histamine content of rat tissue which again is opposite to that which occurs following adrenalectomy

More recently Nilzen[72 74] has reinvestigated this problem utilizing both thyroidectomy and I^{131} administration to suppress thyroid activity

He was able to confirm the results already referred to however the course of allergic encephalomyelitis was apparently an exception being uninfluenced in guinea pigs by these procedures Of further significance was the fact that in guinea pigs sensitized to egg albumin local and general anaphylaxis as well as the formation of precipitin were abolished but the Schultz Dale reaction could still be elicited

There is no clear explanation for these results However there seems to be less controversy as compared with pituitary adrenal effects in that the findings of different investigators are in agreement The balance between thyroid on the one hand and pituitary adrenal on the other has been considered by Long and Miles[41] who attempt some explanation of thes results While the relation of the thyroid to immune mechanisms in animal species is of great interest there is no clear evidence that allergic manifestations of man are influenced by thyroid activity

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39

*Effect of Steroids on Antibody Production in Vivo**

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It has been shown in a number of laboratories that ACTH or cortisone suppresses the formation of circulating antibodies in experimental animals[2, 6, 13, 15, 16, 20] Since little is known about the biochemistry of antibody formation or the mechanism of action of adrenal hormones a satisfactory explanation of these antigen antibody steroid relationships must await more experimental data than are now available Nevertheless it seems reasonable to assume that active adrenal steroids interfere with some step in the synthesis of antibody protein either directly or through a secondary process Whether this function is accompanied by a chemical change in the steroid molecule is an important question Consequently it has been of interest to study steroid metabolism in relation to suppression of antibody by adrenal hormones Among the many problems in this field is the relation of steroid structure to antibody inhibiting properties the effect of ACTH on the circulating adrenal steroids in the species under study and the normal metabolism of these steroids The present report is concerned with some aspects of these questions as they relate to the sheep cell hemolytic antibody system and steroid metabolism in the mouse

Effect of Various Steroids on the Suppression of Circulating Hemolytic Antibody in the Mouse

Early experiments in this investigation were concerned with standardizing a quantitative antigen antibody reaction in the mouse to show a constant cortisone effect against which the activities of other steroids could be compared[15] In general the procedure adopted involved two groups of 10 control mice two groups treated with cortisone and two treated with the test steroid One day after steroid treatment all animals were immunized with a standard dose of sheep cells and bled 6 days later

* These investigations were assisted by grants in aid from the National Cancer Institute of Canada and the Medical Research Division of the National Research Council of Canada The contributions of several research assistants and students in these laboratories should be acknowledged and their names have appeared—or will—on more detailed publications

The sera of each group were pooled and measured for hemolysins by the quantitative rate reaction method of Mayer[13, 14] The averages of duplicate values of test groups were expressed as percentage suppression of

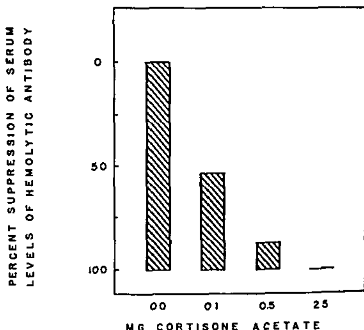


FIGURE 1 Effect of increasing amounts of cortisone acetate on the suppression of circulating hemolytic antibody in the mouse

hemolytic antibody from those of control groups Each set of data was confirmed at least once

It was found that a dose of 2 5 mg of cortisone acetate per mouse consistently gave 100 per cent suppression of circulating hemolysins when a standard dose of 0 1 ml of 1 per cent sheep cells served as antigen by either the intraperitoneal or intravenous routes[15] Figure 1 shows the effect of increasing doses of cortisone acetate on the inhibition of serum levels of hemolytic antibody The 2 5 mg dose of cortisone acetate caused a loss of body weight and a marked atrophy of thymus and spleen Despite these conditions as shown in Figure 2 when the amount of antigen was increased percentage suppression of hemolytic antibody from control levels decreased until substantial amounts of antibody appeared Thus the observed suppression from normal levels of antibody by cortisone proved to be a function of the doses of both hormone and antigen[15]

Similar progressive antibody inhibition was observed with the standard dose of antigen and increasing amounts of corticotropin (ACTH) Also

an elevation of antibody occurred in animals receiving large amounts of ACTH (60 I U per day) when the dose of antigen was increased [8] These results and those from the cortisone experiments suggest that in

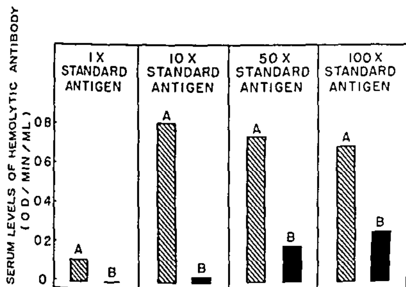


FIGURE 2 Effect of circulating hemolytic antibody levels of increasing amounts of antigen in normal mice and mice receiving a constant amount of cortisone acetate A groups receiving antigen only B groups receiving antigen and 25 mg cortisone acetate per mouse

the system under study a complete block of the antibody producing mechanism probably cannot be accomplished with adrenal hormones if the dose of antigen is sufficiently large

It is well known that adrenal hormones such as hydrocortisone undergo a complex series of chemical changes in the organism [8] It is not clear however whether these changes are associated with physiological functions or whether they represent nonspecific reactions involved with a general breakdown of the molecule in preparation for excretion Thus it is of interest to know the antibody inhibiting properties of a variety of steroids related in structure to the adrenal hormones such knowledge permits the identification of specific chemical changes with loss of activity Accordingly an investigation was carried out to compare the hemolysin inhibiting activities of several steroids including where possible known metabolites of the adrenal hormones The dose of antigen selected for these experiments was such that it permitted 100 per cent suppression of hemolysins from control levels by 25 mg of cortisone acetate The results are given in Figure 5

Effect of Corticotropin and Metabolism of Adrenal Steroids in the Mouse

Before discussing the data in Figure 5 a related study on steroid metabolism in the mouse should be outlined briefly In these experiments mice were bled before and after treatment with ACTH, and the plasma specimens were analyzed for adrenal steroids by dialysis extraction and paper chromatography [19, 1] After elution the steroid zones were measured and characterized by the usual chemical methods [19, 21] Similar procedures were employed to study the metabolism of intravenously infused steroids by analyzing blood and liver samples taken at various time intervals following steroid injections In some instances these *in vivo* experiments were accompanied by *in vitro* studies on isolated tissues and liver cell fractions

During the course of this study it was learned that ACTH caused a

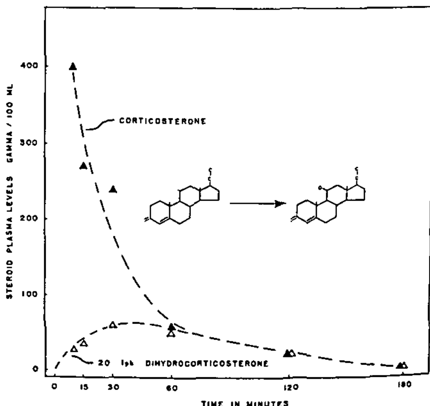


Figure 3 Metabolism of corticosterone in the mouse Corticosterone and 20-alpha dihydrocorticosterone levels in mouse plasma following intravenous infusions of 100 gamma of corticosterone per mouse Plasma pooled from groups of 20 mice

marked increase in the corticosterone levels of mouse plasma and also increased the amounts of a steroid identified as 20 α dihydrocorticosterone.[19] Subsequent *in vivo* infusion experiments showed that 20 α dihydrocorticosterone is an early product of corticosterone metabolism detectable in the blood and liver prior to the appearance of ring A reduced metabolites.[8] Since the 17 hydroxyl adrenal steroids such as cortisone and hydrocortisone could not be found in mouse tissues after treatment with ACTH,[19] it would seem that the primary adrenal corticoid of the mouse is corticosterone.

Figure 3 gives the results of *in vivo* infusion experiments with corticosterone and illustrates the rate of disappearance of corticosterone from the blood and the appearance and disappearance of its C-20 α dihydro derivative. *In vitro* experiments with several organs showed the liver and to a lesser extent the kidney to be the most active in C-20 α reductase

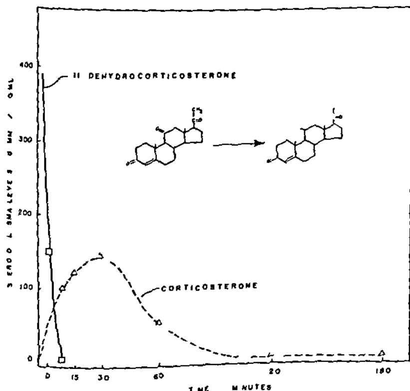


FIGURE 4. Metabolism of 11 dehydrocorticosterone in the mouse. Corticosterone and 11 dehydrocorticosterone levels in mouse plasma following intravenous infusions of 160 gamma of 11 dehydrocorticosterone per mouse. Plasma pooled from groups of 20 mice.

activity[5] Although the β epimer of 20α dihydrocorticosterone could not be found in specimens from the *in vivo* experiments liver homogenates on incubation with corticosterone yielded both the α and β 20 hydroxyl isomers More recent studies have shown that these two enzyme activities can be separated by ultracentrifugation of liver homogenates The α reductase remains in the supernatant at 100 000 g and can be purified by acetone fractionation and dialysis to yield a preparation free of C 11 reductase C 20 β reductase and the ring A reductase systems[5]

Intravenous infusion experiments with corticosterone in the mouse also revealed its conversion to 11 dehydrocorticosterone in the liver[5] However this 11 keto steroid did not appear in the blood On the other hand when 11 dehydrocorticosterone was infused intravenously it rapidly disappeared from the blood and a portion reappeared as corticosterone in both blood and liver Figure 4 gives the plasma levels of corticosterone and 11-dehydrocorticosterone found in these experiments

COMMENTS

In view of the above findings the following comments seem pertinent to the data in Figure 5

Corticosterone (IV) the normal corticoid of the mouse proved to be the most potent substance tested and hydrocortisone (VI) its 17 hydroxyl derivative showed almost equal activity Therefore the presence of a 17 hydroxyl group neither enhanced nor depressed the potency of corticosterone

The activity of 11 dehydrocorticosterone (III) was much less than that of corticosterone (IV) and repeated efforts even at doses of 10 mg per mouse failed to suppress the production of hemolytic antibodies completely[17] The results of infusion experiments with 11 dehydrocorticosterone (III) compared with those of corticosterone (IV) (Figures 3 and 4) offer a possible explanation for this striking difference in activity Some 11 dehydrocorticosterone (III) was converted to corticosterone (IV) in the liver by reduction of the C-11 ketone to a β hydroxyl group The corticosterone thus formed was apparently released by the liver and elevated blood levels of the normal endogenous hormone but in amounts substantially less than those occurring after the infusion of equivalent quantities of corticosterone In view of these findings it was not surprising that 11 dehydrocorticosterone (III) showed less activity than corticosterone (IV) in the quantitative hemolysin test These explanations are not adequate however to account for the approximately equal potencies of cortisone (V) and hydrocortisone (VI) Although these hormones are not normal mouse steroids it has been established that cortisone is reduced to hydrocortisone by mouse liver[5] Further data comparing the effect of

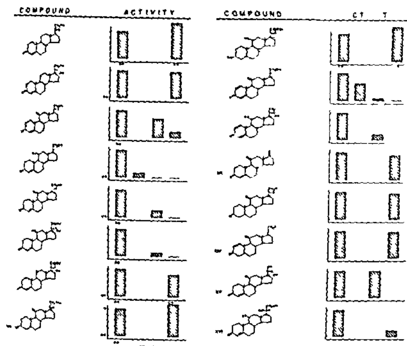


FIGURE 5 A comparison of the effects of the following steroids on the production of circulating hemolytic antibodies in the mouse Each bar represents average values from two groups of 10 mice

I 11 desoxycorticosterone
II 11 desoxy 17 hydroxycorticosterone
III 11 dehydrocorticosterone
IV corticosterone
V cortisone
VI hydrocortisone
VII epi hydrocortisone
VIII tetrahydrocortisone acetate
IX tetrahydrohydrocortisone acetate
X 1 2-dehydrocortisone
XI 1 2 dehydrohydrocortisone
XII adrenosterone
XIII 4 5 dihydrocortisone
XIV 1 2-dehydro-4 5 dihydrocortisone acetate
XV 21 deoxycortisone
XVI 20 beta dihydrohydrocortisone

17 hydroxylation on the rates of steroid enzyme reactions in the mouse may help explain the anomaly that 11 dehydrocorticosterone (III) is less active than corticosterone (IV) but cortisone (V) has about the same activity as hydrocortisone (VI)

The antibody suppressing property of 20 β dihydrohydrocortisone (XVI) is of interest because little is known about the biological properties of the C 20 reduced adrenal steroids [4] If 20 β dihydrohydrocortisone (XVI) oxidized to hydrocortisone (VI) in mouse tissues this could account for the observed activity but efforts to isolate hydrocortisone from mouse blood and liver after intravenous infusions of 20 β dihydrohydrocortisone have not been successful [5] The finding that this steroid

is biologically active in the mouse as regards antibody suppression is of further interest in light of the observation by Long[1] that 20 β dihydro hydrocortisone (XVI) was glycogenic in adrenalectomized mice

The inactive compounds shown in Figure 5 call attention to alterations in chemical configuration which cause an active molecule like corticosterone or hydrocortisone to lose its ability to interfere with the synthesis of antibody protein Reduction of the double bond at C 4–5 (XIII) or reduction of both the double bond and 3 keto group (VIII IX) inactivates the molecule Also reduction of the double bond at C 4–5 and substitution of a double bond at C 1–2 (XIV) gives an inactive steroid. However the addition of an extra double bond at C 1–2 (X XI) does not alter the potency of either cortisone or hydrocortisone Removal of the β hydroxyl group at C 11 (I, II) or substitution with an α hydroxyl group (VII) yielded inactive products Finally, removal of the side chain (XII) produces an inactive molecule as does conversion of the C 21 primary alcohol to a methyl group (XV)

Insofar as the sheep cell hemolytic antibody system in the mouse is concerned although maximum adrenal steroid activity requires a Δ^4 3 keto structure in ring A a β hydroxyl group at C-11 and an α ketol arrangement in the side chain some activity is permitted by a ketone at C 11 and a β glycol side chain If speculation is in order there appear to be at least two biochemical concepts to consider in explaining the role of the adrenal hormone in suppressing antibody formation First the active steroid may function directly at some stage in the synthesis of antibody either by physically blocking an essential mechanism or by participating through one or more of its active groups in some unknown chemical reaction associated with antibody production A second possibility is that the adrenal hormone may interfere with the synthesis of antibody through a secondary effect resulting for example from some primary disturbance in carbohydrate, protein or energy metabolism Whatever the final explanation may be the fact remains that antibody synthesis will take place in a high concentration of antigen in the system under study in spite of the general catabolic effects induced by large amounts of adrenal hormone

In conclusion it should be emphasized that adrenal steroid secretions and metabolic reactions of the individual hormones may vary with the species[6, 18] Similarly immunological experiments may yield different quantitative data depending upon the nature and quantity of antigen and the species Thus the results and conclusions expressed in this paper apply only to the sheep cell hemolytic antibody system in the mouse and these antigen antibody steroid relationships may or may not be applicable to other species

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40

*Cortisone and Lymphoid Tissue in Relation to Hypersensitivity**

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Over the past fifty years a number of facts have become apparent suggesting a relation of lymphoid cells to immunity and thereby to hypersensitivity. Early experiments by Hectoen[2] revealed that x-radiated animals were unable to form antibody of the classic type. Somewhat later Murphy[3] demonstrated that x-radiation also caused a loss of presumptive immunity—unassociated with measurable antibody—in the form of diminished resistance to experimental infections with tuberculosis and to grafting with homologous tissue.

The impaired immune responses observed were suggested by these authors to be a consequence of the destructive action of the x-rays upon lymphocytes. Quite understandably this view was challenged by many who thought that x-radiation causes widespread damage to structures other than lymphoid tissue, notably the bone marrow and the reticulo-endothelial system. However, over the past ten years a number of observations have given further support to the idea that the integrity of lymphoid tissue is important for the proper function of the immune mechanism.

Comparing the effect of various nutritional deficiencies upon the state of lymphoid tissue and upon the development of immune responses, it was found that pyridoxine deficiency caused nearly complete loss of lymphoid cells associated with an inability to form circulating antibody.[13] Other nutritional deficiencies of comparable severity caused less atrophy of lymphoid structures and no impairment of antibody responses.[4] With the aid of a pyridoxine antagonist[8] it was found that in rats lymphoid atrophy and immune paralysis could be produced in 2 weeks. Also in previously immunized animals the anamnestic response could be abolished by the same treatment. From experiments in passively immunized animals deficient in vitamin B₆ it was seen that the degradation of antibody proceeded at a normal rate, indicating that antibody formation was

* From the Merck Institute for Therapeutic Research, Rahway

impaired during pyridoxine deprivation Unlike the results in radiation injury, the bone marrow remained intact and the reticuloendothelial system as judged by its ability to phagocytize foreign material appeared unharmed Subsequently it was observed that the administration of two other agents causing striking loss of lymphoid cells — nitrogen mustard[7] and an antagonist of pteroyl glutamic acid[6] — was also followed by inhibition of antibody formation These two substances like x radiation caused bone marrow damage in addition to lymphocytolysis Finally less than ten years ago when cortisone became available in sufficient quantities and its properties could be studied in greater detail it became evident that in addition to its numerous still poorly understood actions, it was a potent but relatively harmless lymphocytolytic agent[16] In addition to its therapeutic effect in a number of conditions long suspected to be allergic etiology[16] it also suppressed immunity and certain reactions of experimental hypersensitivity[9] Like deoxypyridoxine it spared the granulocytic *and erythroid elements of the marrow and even appeared to enhance* the phagocytic activity of reticuloendothelial cells It was also seen that low antibody levels resulting from cortisone treatment were not associated with increased degradation of antibody protein[1] and were therefore a result of impaired antibody formation

From all this it appears that over the past decade Hectoen s hypothesis has gained much support although any causal relation between lymphocytes and the formation of immune body remains unknown

Murphy s findings regarding the facilitation of homografts and lowering of resistance to infection with tuberculosis through x radiation have also found their counterpart in experiments in which lymphoid atrophy was produced by desoxypyridoxine nitrogen mustard or cortisone[10] In these types of immunity which still defy the exacting methods of immunochemistry some evidence has been acquired to suggest a direct participation of lymphoid cells

Much of the early work with grafted tumors has led to the recognition that homografts of tumor tissue like grafts of homologous normal tissue are truly compatible only among genetically identical members of a given strain This situation except between identical twins exists only among mice of a highly inbred strain Other homografts save for a number of exceptional instances are rejected and lead to the development of an even greater resistance on subsequent grafting This phenomenon of acquired resistance to homografts was assumed by most of the early observers to be related to some form of immunity Much morphological evidence obtained by Leo Loeb Murphy and Kidd pointed to the lymphocyte as being instrumental in the process of homograft rejection Finally it could be shown by Kidd and Toolan[8] that when lymphocytes taken from an immune animal that is one in which a tumor graft has regressed are

mixed with tumor cells and the mixture is then inoculated into a susceptible host, the immune lymphocytes will prevent the tumor cells from growing. Most interestingly the immune activity carried by these cells could not be extracted and was observed only with uninjured viable cells. Sera of mice in which tumor grafts had regressed failed to influence the growth of tumor cells in susceptible mice.

Much earlier immune activity of a type resembling that just described had been observed by Landsteiner and Chase in the delayed or tuberculin type of hypersensitivity. Here also viable cells were capable of transmitting the immune reactivity from one animal to another while cell extracts or serum failed to do so. The demonstration that both these types of immunity associated with sensitized cells are very quickly suppressed by lymphocytolytic agents like cortisone points to the possibility that the suppression of these two forms of hypersensitivity may be a consequence of lymphocyte destruction.

In contradistinction to the prompt effect of cortisone in the above conditions suppression of established hypersensitivity of the immediate type (Arthus anaphylaxis) requires treatment for a duration outlasting the lifetime of preformed antibody.

I shall now summarize some experiments dealing with the action of lymphocytolytic agents upon the immunity or hypersensitivity which determines the incompatibility of homografts. Under the conditions of these experiments the fate of homotransplants appears dependent upon the state of lymphoid cells both in the donor and the recipient of a homograft. Accordingly lymphocytolytic agents including cortisone administered to the donor diminish the antigenicity of the homograft while the same treatment to the grafted animal diminishes the recipient's resistance.

Our interest in immunity against homografts started with observations made during a comparison of the growth of tumor transplants in mice and rats deprived of single factors of the vitamin B group.

It was found that pyridoxine deficiency caused striking regression of lymphosarcoma transplants. However the regression was in no case complete and when the animals in order to be kept alive were refed with pyridoxine the tumors recurred and killed the host.[8] In contradistinction lymphosarcoma bearing rats and mice deprived of vitamin B_2 showed a much less spectacular slow regression of the tumors which after about 4 weeks was complete and was not followed by recurrence when the animals were returned to a normal diet.[15] Moreover when it was attempted to reinoculate these animals with lymphosarcoma cells they proved resistant to the second tumor graft.

From the studies previously mentioned we had learned that pyridoxine deprivation causes loss of normal lymphoid tissue and depression of im

mune responses while riboflavin deficiency had no such effect. It therefore appeared quite likely that the complete regression of the lymphoma was aided by some form of immunity directed against the tumor transplants. This possibility was tested in tumor bearing rats deprived of riboflavin by *complicating the riboflavin deficiency with a number of conditions* known to suppress lymphoid tissue and immunity. In Figure 1 it can

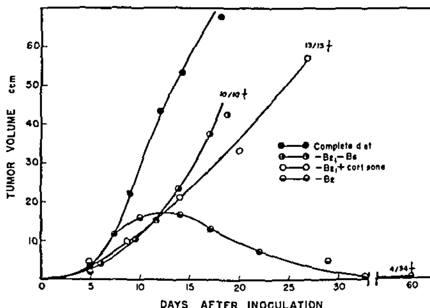


FIGURE 1. Loss of immunity prevents tumor regression due to vitamin *B* deficiency.

be seen that impairment of immunity through administration of desoxypyridoxine or cortisone abolished the curative effect of riboflavin deficiency. Similar observations were made when riboflavin deprivation was complicated by x radiation or nitrogen mustard treatment. This of course led to the recognition that the observed tumor regression was largely due *to immunity against a homograft* and was only *in part related to the riboflavin deprivation*.

Subsequently the nature of the immunity which developed after tumor regression was examined further. Some of the findings are summarized in Figure 2. Confirming Kidd and Toolan's observations, viable lymphoid cells of rats resistant to renewed inoculation when mixed with tumor cells and then injected into susceptible rats prevented the growth of the tumor cells. *Injury to the sensitized cells — even as mild as freezing or heating to* 45° C — *abolished their immune activity*. *However*, in the same animals measurable immune activity was also demonstrable in the serum

The properties of such sera were studied and were found to differ distinctly from those of rabbit immune sera against rat lymphosarcoma or from those of any other immune serum of the classic type

The immune activity of the serum was associated with the gamma globulin fraction was stable to freezing or heating up to 60 C and when injected into susceptible rats conferred passive immunity over about

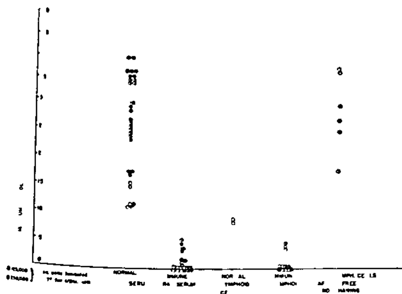


FIGURE 2 Inhibition of growth of lymphosarcoma cells in susceptible rats following the addition of serum or of "sensitized cells from immune rats to the inoculum Each point in the graph represents the average tumor volume in 6 susceptible male rats 14 days after inoculation

2 weeks No difference in blood groups could be detected in the strain of rats employed (Sprague Dawley) and active immune sera neither caused nor inhibited agglutination of rat erythrocytes *In vitro* the immune sera reacted in no measurable way with viable lymphosarcoma or other viable rat cells Even after prolonged incubation of sera with suspension of lymphoma cells the latter when separated from the serum and injected into susceptible rats proved viable and the sera exhibited no detectable loss of immune activity (Table I) In contrast to immune sera against tumor transplants previously observed by Lumsden and by Gorer ours could not be absorbed with rat erythrocytes or with viable normal lymphoid or lymphosarcoma cells However when lymphosarcoma or normal lymphoid cells injured by freezing or mild heating were incubated with highly active immune sera this contact with disrupted cell structures removed the immune activity from the sera (Table II) The same

TABLE I FAILURE OF IMMUNE SERUM TO CAUSE LOSS OF VIABILITY OF TUMOR CELLS IN VITRO

250 000 Lymphosarcoma Cells *Incubated with*	After 24 Hours Separated and *Resuspended with*	Tumor Volume [illegible] in cc *Aver*	*Range*	No of Takes
Immune serum	Immune serum	1 9	0– 6 6	3/6
Immune serum	Normal serum	24 6	0–38 6	5/6
Normal serum	Normal serum	23 5	5 3–43 8	6/6

TABLE II LOSS OF ACTIVITY OF IMMUNE SERUM FOLLOWING INCUBATION WITH EXTRACTS OF NORMAL LYMPHOID TISSUE

				CM³ — Tumor Vol *at 14th Day*		
Group	Immune Serum	Intact Cells	Extract of Cells	Aver	Range	No of Takes
1	+	—	—	6 5	0–37 4	7/8
2	+	Spleen	—	5 7	0–18 1	6/8
3	+	—	Spleen	20 4	0–41 5	7/8
4	+	Thymus	—	8 2	1 5–35 3	8/8
5	+	—	Thymus	16 4	7 2–36 0	8/8
6	—	—	Spleen	24 1	2 4–33 4	7/7
7	—	—	Thymus	26 7	15 5–52 5	8/8
8	—	—	—	38 9	19 8–54 7	7/7

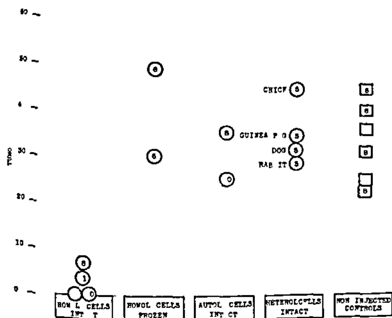
Groups 1 2 4 significantly different *from groups* 3 5 6 7 8 P < 0 02

loss of immune activity from serum followed the exposure to saline – or to ether extracts from a variety of animal tissues including those of heterologous and autologous origin Thus whatever the nature of the reaction between the rat anti rat lymphosarcoma serum and the moiety which causes the loss of its activity may be it does not take place on the cell surface or across the uninjured structures of the cell However cell injury appears to make accessible some agent which abolishes the activity of immune sera of this type

In view of these findings it seems probable that the loss of immune activity through cell injury which characterizes the sensitized cells of Kidd may be explained in a similar fashion Since it was found that both the immune activity and its neutralizing counterpart reside in the same cells it seems likely that in intact cells they are spatially separated from each other while following cell injury the immune factor is exposed to the agent which abolishes its activity

It has been known for a long time that injections of various homologous

tissues may give rise to acquired resistance aga n t homologo s tu n or grafts Comparing various tissues with regard to th s ant ge c property appeared that suspensions of viable lympl o d cells re tl n tl er viab e homologous tissues gave rise to immunity aga t n olo ous e plast c and normal tissue al ke Homologous tiss ies n e l l fre zing or b exposure to 45° C for 15 minutes exerted no n g a ti n Heterologous or autologous tissues also proved dev d f a t cn c ty n th s respect (Figure 3) Thus organ specific antigens cre n t a leter



F GURE 3 Res stance to grafting h lymphosarcoma follo ng a s ngle n ec tion of ntact homologous lympho d cells

mining factor in th s immunity to viable homologous cells Rather it appeared that acquired res stance to homologous t ssue grafts was related to one and the same ant gen c component d stributed over most t ssues of the same animal but differing from one rat to another and thereby characterizing the animals b ological individual ty

Surpr singly it was also seen that t ssues from donors treated with cortisone or other lymphocy tolytic agents exh b ted a striking d m nu tion of their antigen c property This could be demonstrated in t o fash ons immunization w th viable tissues from such donors proved neffec tive (F gure 4) and skin from donors treated v th cortisone or n trogen mustard v as rejected much later than that of untreated donors [4]

It was seen further that injections of suspens ons of viable cells from

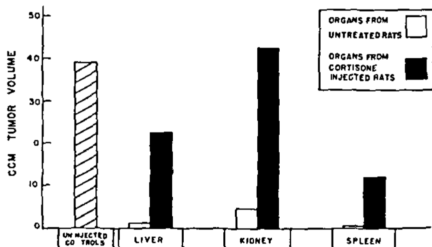


FIGURE 4 Loss of homoio antigenicity from tissues of cortisone injected rats

homologous spleen or liver in the recipient rats gave rise to the sensitized cells of Kidd [3] This was demonstrable in a semiquantitative fashion by the local inhibitory effect of the sensitized cells upon the growth of homologous tumor cells in susceptible rats As mentioned earlier injections with injured homologous cells produced no resistance to homografts and did not give rise to sensitization of the recipient animals lymphoid cells Sera of rats immunized with normal homologous cells so far have failed to exhibit measurable immunity It appears likely that the immune sera observed following tumor regression are the result of a more thorough immunization by large masses of slowly regressing homologous tissue

If one should care to bring together the few facts which have been recognized from these experiments this may be done in the following manner Contrasting in the immune system in question the relatively high specificity of the antigen — viable homologous tissue — to the seemingly nonspecific reactivity of immune serum with extracts of autologous homologous or heterologous tissue a possible working hypothesis suggests itself it is conceivable that the antigenic moiety which gives rise to immunity against homologous tissue is present in most viable cells and represents a complex which in its entirety is specific for each individual of a given species This complex is labile and is denatured following injury to the cell but a haptenic component of the antigen which is alike for all species persists This hapten is still able to react with the homologous immune serum but is not antigenic

From the experimental evidence presented it appears that in relation to

the immunity against homologous tissue Lymphoid cells play an important dual role They usually seem to be the main carriers of the immune principle in question which unlike classic antibody is dependent for its persistence upon the integrity of the lymphoid cells Thus it seems plausible that cortisone or other lymphocytolytic agents should suppress immunity to homologous tissue and possibly other forms of hypersensitivity as well

In addition the moiety responsible for the antigenicity of homologous tissue in some way also appears dependent upon the presence of adequate numbers of lymphoid cells If lymphoid atrophy is produced in a donor of a homograft its tissues are rendered less antigenic and consequently more compatible to the recipient

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GENERAL DISCUSSION

EDWARD H KASS (Boston Massachusetts) I had the opportunity last night to discuss a few of these matters with Dr Darrach and thought that others here would be interested in some of the implications of the problem of steroid differences as we discussed it

The general biologic problem that is posed is this If corticosterone is the sole or predominant steroid that is manufactured by certain species of animals and if hydrocortisone is predominately synthesized by others is there any biologic advantage or disadvantage to one or the other hormone? In a narrower sense are the differences in the nature of the hormonal output sufficient to explain some of the differences that have been observed between administration of ACTH and administration of either of these purified steroids?

I suppose our group has contributed as much as any to the confusion that reigns with respect to this problem We were able to show a few years ago that corticosterone differed from hydrocortisone in that corticosterone did not depress resistance to infection whereas hydrocortisone did so Corticosterone did not cause lymph node atrophy depression of antibody production or depression of the RNA response that follows antigenic stimulation whereas hydrocortisone did all of these things It is ordinarily held that hydrocortisone produces more glycogenic deposition in the liver than does corticosterone

The difficulty with these observations is that the hormone had been given subcutaneously in all of these experiments In nature the hormones are infused at a relatively constant rate What would happen if we tried to mimic this system?

We have used an endotoxin system in which endotoxin mixed with steroid can be given intracardially Using this system one finds that corticosterone is just about as active as hydrocortisone and one can construct a new hierarchy of steroid activity in which turnover from the site of tissue deposition plays very little role and in which presumably a much closer relationship between structure and function can be worked out

I cite this because it destroys what was a very nice hypothesis that we advanced a number of years ago with respect to differences between corticosterone and hydrocortisone and also because I think it does illustrate the many pitfalls one can get into in attempting to reproduce what happens in nature by an essentially artificial laboratory situation Thus unless experiments are constructed to circumvent this problem it must be concluded that many studies of the biologic effects of corticosteroids measure simultaneously tissue turnover and true biologic activity It is

conceivable that many important activities are not recognized because of accelerated tissue turnover

DR DARRACH I should comment on one point not mentioned in the presentation and that is the need for uniform absorption of steroid from the site of injection This presented a difficult problem and required the depots to be cut out and analyzed at the end of the experiment to be sure all the steroids had disappeared In the experiments described the subcutaneous injections were made with microfine suspensions of the steroids in Tween 20 With this technique we have been unable to find the administered compounds remaining in the depots at the end of the experiment

KINGSLEY M STEVENS (West Point Pennsylvania) We have a system that might be of interest to Dr Darrach

This is a rabbit spleen system using bovine gamma globulin as an antigen in a completely *in vitro* system augmented by endotoxin initiation As part of another study we have studied sodium prednisolone phosphate a soluble steroid At concentrations as low as 2×10^{-5} M you can get 75 per cent depression of antibody formation At 2×10^{-4} M no antibody is formed The effect is almost immediate and irreversible by washing

We rather expected that if we added antigen first we might have little effect since steroids don t have as much effect on the secondary antibody response as on the initiation phase However we found both phases equally sensitive We get the same effect with nitrogen mustard except the concentration is somewhat different 10^{-4} M mustard produces the same depression as 10^{-5} M prednisolone

RUDOLF E WILHELM (Detroit Michigan) Dr Stoerk you postulated that steroids seem to exert their effect as antilymphocytic agents the role of lymphocytes in allergic responses is also supported by Dr Inderbitzin who yesterday showed that the administration of antilymphocyte serum caused the reduction of tuberculin skin test reactivity

We have done similar studies by administering antilymphocyte serum and have found that this resultant experimental lymphopenia also reduced the contact type sensitivity in dinitrochlorobenzene sensitive guinea pigs this effect correlated also with the peripheral lymphopenia and the lymphopenia in the histological sections of contact type skin lesions of such animals

The Role of Mycobacteria in Allergic Manifestations

Chairman HERBERT C STOERK MD (*Rahway New Jersey*)

Chairman's Remarks

The papers in this afternoon's session deal with the enhancement of experimental hypersensitivity with the aid of Freund adjuvants Since the time of Pasteur a variety of substances have been admixed to antigens in order to intensify immune responses In recent years an adjuvant emulsion developed by Freund and McDermott[2] has proven most useful in a great number of immunological investigations The basic component of this adjuvants mixture consists of paraffin oil into which, with the aid of Aquaphor water soluble or suspended antigens are mixed together with heat killed tubercle bacilli It has been known for a long time that dead tubercle bacilli cause a tissue reaction quite similar to that typical of tuberculous infections Accordingly intramuscular or subcutaneous injections of the adjuvants mixture give rise at the site of injection to extensive inflammatory lesions containing numerous epithelioid cells which are hardly distinguishable from tuberculous granulation tissue Dissemination of the emulsion which contains fragments or soluble components of the tubercle bacilli frequently causes extensive granulomatous lesions in lymph nodes regional to the site of the injections However miliary and larger foci of the granuloma may also be found in lung indicating that some dissemination occurs over the blood stream In addition to these local reactions a striking systemic change concerns the lymphoid tissue of animals injected with Freund adjuvants Lymph nodes and spleen are grossly enlarged and exhibit hyperplastic lymph follicles with conspicuous germinal centers These changes are comparable to those first described by Hellman[5] in animals intensely immunized without the use of adjuvants

The enhancing effect of the Freund adjuvants upon antibody formation has been demonstrated for a number of immune systems From a quantitative study comparing guinea pigs immunized with egg albumen in the complete adjuvants mixture with guinea pigs immunized with a similar emulsion lacking tubercle bacilli it was found that antibody N concentrations in the sera of the animals immunized with the antigen in complete Freund adjuvants were about 5 times higher than that of the controls At

the present state of knowledge concerning the mechanism of antibody formation attempts to explain its enhancement seem rather fruitless Some investigators have thought that the epithelioid cells contribute in the increased immune body production while others prefer to believe that the retention of antigen by the granuloma with a subsequent gradual release, accounts for a more effective immunization Also in view of findings indicating that lymphoid atrophy seems invariably associated with an inability to form antibody it comes to mind that generalized lymphoid hyperplasia may account for enhanced antibody formation

Since enhancement of immunity is expected to entail enhancement of hypersensitivity it does not seem surprising that the usefulness of adjuvants has become most spectacular in experiments dealing with the enhancement of certain experimental diseases presumed to be of allergic etiology The four papers included in this section of the symposium deal with this important subject

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*The Adjuvant Effects of Mycobacterial Cells and Fractions**

ROBERT G WHITE MA DM (Oxon)
(London England)

The addition of killed tubercle bacilli to the water in oil mixture which contains an antigen in its aqueous phase will promote a wide variety of immunological results Freund and McDermott[14] first used such adjuvant mixtures for the production of high titers of circulating antibody They have also been widely used for the induction of skin sensitization of the delayed type to simple chemicals[16] and protein antigens such as tuberculin[6] and ovalbumin[8] for the production of acute disseminate encephalomyelitis with homologous or heterologous brain[5 17] for the production of aspermatogenesis with homologous testicular tissue[13] for the induction of acinar damage and granulomatous infiltration of the thyroid gland with homologous thyroid tissue[19] and for the induction of immunity to a wide variety of animal parasites

With R G S Johns and L Bernstock of the London Hospital England I have been concerned with the role of these added tubercle bacilli in potentiating the antibody production to ovalbumin The influence of *Mycobacterium tuberculosis* (H37Rv) in oil adjuvant mixtures containing ovalbumin in increasing serum antibody was first accurately assessed by Fischel Kabat Stoerk and Bezer[2] We have been able to study the activity of whole bacilli and chemical fractions prepared for us by Dr E Lederer of l Institut de Biologie Physicochimique Paris

When a guinea pig is inoculated into a hind foot pad with 5 mg of ovalbumin in water in oil emulsion of Arlacel A and Bayol F containing 200 μg of killed tubercle bacilli and a control animal is treated similarly except for the omission of the mycobacteria striking differences are apparent in their behavior over the following 3 weeks Although no difference can be seen for the first several days from the beginning of the second week the animal receiving the tubercle bacilli shows great

* The work was supported by grants from the Central Research Fund London University the Medical Research Council and the Waksman Foundation of France

enlargement of the injected foot which from being spongy soft becomes firm with cyanotic surface which later becomes denuded of hair and ulcerated. At the same time the lymph nodes of the homolateral flank become enlarged and are obviously several times the size of those of the control. A section of the injected foot and of the regional lymph node yields a picture of intense granulomatous cellular proliferation consisting predominantly of macrophage elements of epithelioid cell type. Also when such animals are tested at 19 days with an intracorneal injection of ovalbumin 48 hours afterwards the cornea is grossly thickened and opaque grayish white throughout most or the whole of its extent. The control similarly tested shows hardly any abnormality or slight opacity near the site of the needle puncture. Thirdly the animal's serum contains 300 to 600 μg N per milliliter of antiovalbumin whereas the control has somewhere between 0 and 00 μg N per milliliter. We have usually tested the various chemical fractions or bacilli by injecting a single dose of 200 μg in Arlacel Bayol mixture containing 5 mg of ovalbumin into each guinea pig.

In this way it is possible to show activity in respect of the morphological changes as described above the production of an increase in serum antiovalbumin and corneal hypersensitivity to ovalbumin with a wide variety of whole mycobacteria. Thus several strains of human and bovine types of *Myco tuberculosis*, an avian type saprophytic mycobacteria such as *Myco phlei* and *smegmatis* and several *Nocardia* (*asteroides*, *brasiliensis*, and *rhodochrous*) proved active.

Extraction of *Myco tuberculosis* with ether alcohol mixture followed by chloroform in accordance with the procedure of Anderson[2] and Asselineau and Lederer[6] yields after fractionation four waxy substances — waxes A B C and D. The ether alcohol soluble material or crude phosphatide fraction (which includes wax A) of *Myco tuberculosis* (H37Rv) was tested and found quite inactive. It neither caused any morphological changes different from those of the controls nor raised serum antiovalbumin levels nor altered the corneal reaction to ovalbumin. Wax C cord factor (or trehalose dimycolate) from wax C, and phthiocerol diacetate were all found to be similarly inactive in these respects. *However the so called purified wax fraction* (Anderson) is active and so are wax D fractions prepared from several different human types of *Myco tuberculosis*. Briefly purified wax fraction (Anderson) is obtained from the ether ethanol residue after extraction with chloroform and after treating the extract with ether methanol mixture. Wax B is the soluble portion. Wax C is the hot acetone extract of the purified wax and wax D the insoluble residue after extraction with boiling acetone. Before testing the wax D fractions were dissolved in ether and centrifuged at low temperatures to separate as far as possible bacterial

debris the wax D being then precipitated with methanol centrifuged and dried *in vacuo* Reference to Table I shows that wax D fractions of the human strains Test Canetti and H37Rv are active However several wax D fractions of bovine type *Myco tuberculosis* were tested and found inactive in all respects Thus three samples from the strain B C C two samples from Marmorek and one from Dupre proved inactive Also wax D from the avian type 80 and from two saprophytic types *smegmatis* and *phlei*, proved inactive

It is interesting to compare the activity of the whole bacilli and one of the active wax D fractions The results given in Table II show that equal weights of whole heat killed *Myco tuberculosis* and of a wax D

TABLE I Effect of Mycobacterial Fractions on Antibody Levels and Corneal Hypersensitivity to Ovalbumin

Number in Group	Fraction and Dose	Reference Number	Antiovalbumin (µgN/ml Serum) Mean	Range	Corneal Reaction*
5	Phosphatide H37Rv	WL11	114	42 201	0 0 0 0 0
5	human controls	—	87	39-101	0 0 0 0 0
5	Wax C Canetti	WL1	77	29-169	0 0 0 0 0
5	human controls	—	158	100 205	0 0 0 0 0
4	Cord factor Brevannes	WL22	120	0-252	0 0 0 0
4	human controls	—	70	20 153	0 0 1 0
5	Phthiocerol diacetate	WL3	105	62 199	0 0 0 0 0
4	controls	—	157	100 195	1 0 0 1
4	Purified wax H37	OAI	4[illegible]	345-555	3 3 3 3
4	human controls	—	155	78 276	0 0 0 0
5	Wax D Test	WLO	660	540 762	—
5	human controls	—	187	117 278	—
5	Wax D Canetti	WL2	315	140 463	3 3 3 3 3
4	human controls	—	126	100 200	0 1 0 1
5	Wax D H37Rv	WL28	511	356-800	3 3 3 3 3
5	human controls	—	127	55 245	0 1 0 0 0
5	Wax D B C G	WL7	91	8 195	0 0 0 0 1
5	bovine controls	—	83	31 152	0 0 0 0 0
4	Wax D Marmorek	WL18	170	44 263	0 0 0 0
4	bovine controls	—	136	33 313	0 0 0 0
5	Wax D Dupre	WL26	241	61 310	0 1 0 0 1
5	bovine controls	—	197	125 315	0 1 0 0 0
4	Wax D 802 avian	WL27	152	90 262	0 1 0 0
5	Wax D *M smegmatis*	WL2?	64	10 125	0 0 0 1 0
5	controls	—	102	18 138	0 0 0 0 0
5	Wax D *M phlei*	WL25	98	37-178	0 0 0 0 1
5	controls	—	99	20 176	1 0 0 1 0

* Eye reactions at 48 hours after intracorneal injection of ovalbumin 1 = slight local opacity of cornea around site of needle puncture 2 = moderate opacity of most of cornea 3 = dense grayish white and thickened cornea

[illegible] residue possibly presents a structure somewhat [illegible] wax D preparations of human type *Myco tubercu* [illegible] in the preparation of the bound lipids is then to [illegible] off the glycolipid (without amino acids) from [illegible]

[illegible] the study of the activity of tuberculolipid fractions is [illegible] with small numbers of intact bacillary bodies since [illegible] be maintained that the activity of any particular fraction [illegible] activity of such contaminants By the method of [illegible] stained smears small numbers of bacilli were found to [illegible] the bacillary fractions and were estimated at about 10[4] [illegible], or less than 01 per cent That these small numbers [illegible] could explain the adjuvant properties of chemical frac [illegible] for the following reasons First the activity of wax D [illegible]*losis* Canetti has been shown to be at least of the same [illegible] heat killed intact bacilli Secondly, the wax D fractions [illegible] type of *Myco tuberculosis* Marmorel and of *Myco* [illegible] and *Myco phlei* are ineffective though they too are similarly [illegible] with trace amounts of intact bacillary bodies and it is [illegible] that bacilli from which these are derived are active

[illegible] H L Marshall and I have tested the range of Dr Lederer's [illegible] fractions for their ability to promote lesions of allergic [illegible] in guinea pigs injected with homologous brain The latter [illegible] aseptically from a healthy normal guinea pig and homo-[illegible] immediately in 1 per cent phenol saline This emulsion which [illegible] the dose of 16 mg weight of brain tissue was injected in [illegible] mixture into a hind foot pad The mycobacterial fractions [illegible] were added to the Bayol Animals were observed for periods [illegible] days for the development of encephalitis When such symp[illegible] (1 to 22 days) they were usually followed by a rapid [illegible] over 1 to 3 days to a moribund state The results are shown [illegible] alongside the results of the testing of the same fractions (in [illegible] groups of animals) for activity in increasing serum antibody [illegible] in producing corneal sensitization to ovalbumin The recorded [illegible] animals with encephalitis included only those cases with [illegible] neurological signs and in whom the presence of typical lesions [illegible] confirmed by histological examination

The results show that several different wax D fractions extracted from [illegible] human types of *Myco tuberculosis* proved active Successful pro[illegible] of encephalitis by purified wax and wax D fractions of *Myco* [illegible] has been reported previously[21] although large doses were [illegible] required and some fractions were unaccountably inactive [illegible] several reports[1, 8, 9] of failure with wax fractions We have

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4	human controls	—	70	20–153	0 0 1 0
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4	controls	—	157	100–195	1 0 0 1
4	Purified wax H37	OA1	460	345 555	3 3 3 3
4	human controls	—	155	78 276	0 0 0 0
5	Wax D Test	WLO	660	540–762	—
5	human control	—	187	117 278	—
5	Wax D Canetti	WL2	315	140–463	3 3 3 3 3
4	human controls	—	126	100–200	0 1 0 1
5	Wax D H37Rv	WL28	531	356 800	3 3 3 3 3
5	human controls	—	127	55 245	0 1 0 0 0
5	Wax D B C G	WL7	91	8 195	0 0 0 0 1
5	bovine controls	—	89	31 152	0 0 0 0 0
4	Wax D Marmorek	WL18	170	44–263	0 0 0 0
4	bovine controls	—	136	33 313	0 0 0 0
5	Wax D Dupre	WL26	241	61 330	0 1 0 0 1
5	bovine controls	—	197	125 315	0 1 0 0 0
4	Wax D 802 avian	WL27	152	90–262	0 1 0 0
5	Wax D *M smegmatis*	WL29	64	10–125	0 0 0 1 0
5	controls	—	102	18 138	0 0 0 0 0
5	Wax D *M phlei*	WL25	90	37 178	0 0 0 0 1
5	controls	—	99	20–176	1 0 0 1 0

* Eye reactions at 48 hours after intracorneal injection of ovalbumin 1 = slight local opacity of cornea around site of needle puncture 2 = moderate opacity of most of cornea 3 = dense grayish white and thickened cornea

TABLE II A COMPARISON OF THE ACTIVITY OF HEAT KILLED MYCOBACTERIUM TUBERCULOSIS CANETTI AND A WAX D FRACTION FROM THE SAME ORGANISM

Number in Group	Fraction and Dose	Antiovalbumin (μg N/ml Serum) Mean	Range	Corneal Reaction
4	200 μg Canetti bacilli	509	238–715	3 3 3 2
8	40 μg Canetti bacilli	384	196–575	3 2 3 3 2 2 2 1
8	8 μg Canetti bacilli	130	58–204	1 0 0 0 2 0 0 0
4	1 6 μg Canetti bacilli	120	94–138	0 0 0,0
4	40 μg Canetti wax D	493	229–623	3 3 3 1
8	8 μg Canetti wax D	203	55–285	0 0 0 0 0 2,1 0
11	Controls	100	31–188	0 0 0 0 0 0 0 1 0 1 0

from the same strain Canetti both show about the same order of activity Both are certainly active at doses of 40 μg and over Possibly there is some indication of activity with wax D at a dose of 8 μg There is no evidence of activity with 8 μg of whole bacilli Therefore it is possible to say that wax D of the human strain Canetti is at least as active weight for weight as heat killed whole bacilli

In order to provide information complementary to that derived from studies of chemical fractions the activities of bacilli which had been exhaustively extracted with ether alcohol and chloroform were tested As shown in Table III, when neutral solvents were used the bacillary residues were found to be highly active, whether they were derived from a human strain ($H_{37}Rv$) or from bovine strains (Marmorek and B C G) Anderson Reeves and Stodola [3] showed however that extraction of bacilli with neutral solvents still left unextracted the 'firmly bound lipids When extracted with solvents at acid pH (1 per cent HCl) the residue from $H_{37}Rv$ was found inactive in all respects It is also interesting to note that dried whole bacilli of *Myco phlei* and *Myco smegmatis* both proved highly active in all respects in spite of the fact that wax D fractions prepared from both organisms proved inactive

Before attempting the interpretation of these results let us recall the chemical structure of the wax D fractions Hydrolysis of wax D of human strains yields about half its weight of a mixture of mycolic acids (high molecular weight hydroxy acids with the formula $C_{88}H_{176}O_4$ [7]) and about half of a nitrogenous polysaccharide (molecular weight 6000 to 9000) The polysaccharide contains three sugars arabinose galactose and mannose and three amino acids alanine L glutamic acid and meso α ε diaminopimelic acid [4, 5] According to Asselineau and Lederer [7] the three amino acids form a peptide which is linked to the polysaccharide

TABLE III Effect of Various Whole and Fat extracted Mycobacteria on Antibody Levels and Corneal Hypersensitivity to Ovalbumin

Number in Group	Material for Test	Reference Number	Average Serum Antiovalbumin (contemporaneous control figure in brackets) µg N ml Serum	Corneal Reaction at 48 Hours (contemporaneous controls in brackets)
4	200 µg heat killed *M tuberculosis*	WL9	509 (147)	3 3 3 2 (0 0 1 1)
3	500 µg acid-delipidated *M tuberculosis*	WL17	134 (147)	0 1 0 (0 0 1 1)
5	200 µg neutral solvent extracted *M tuberculosis* human H37Rv	WL31	395 (62)	3 3 3 3 3 (0 0 0 0 0)
5	200 µg neutral solvent extracted *M tuberculosis* bovine Marmorek	WL30	441 (67)	3 3 3 3 3 (0 0 0 0 0)
5	200 µg dried bacilli *M phlei*	WL32	322 (124)	3 3 3 3 3 (0 0 0 1 1)
5	200 µg dried bacilli *M smegmatis*	WL33	400 (124)	3 3 3 3 3 (0 0 0 1 1)

Wax D fractions of bovine and avian types of *Myco tuberculosis* and of saprophytic mycobacteria do not contain any amino acids and thus have the general structure mycolic acid polysaccharide The polysaccharides contain the same three sugars arabinose galactose and mannose

In the present state of our knowledge it is tempting to attribute the activity of the wax D of human strains to the presence of the above mentioned tripeptide linked to the glycolipid Here it should be added that the hydrolysis products of the active wax D of the human strain Canetti (i e mycolic acid or methyl mycolite and the peptide containing polysaccharide) are inactive in all respects and on this evidence it seems that the whole complex is essential for activity

It remains to be explained why neutral delipidated bacilli of human type *Myco tuberculosis* and the whole and neutral delipidated bovine type of *Myco tuberculosis* are also active These delipidated bacilli still contain the firmly bound lipids and they all contain the above mentioned three amino acids alanine glutamic acid and diaminopimelic acid Moreover the work of Cummins and Harris [10, 11] shows these to be the major nitrogenous components of the bacterial cell wall not only of mycobacteria but of the corynebacteria and the nocardia In other words

the neutral delipidated cell residue possibly presents a structure somewhat similar to that of the wax D preparations of human type *Myco tuberculosis* Acid hydrolysis in the preparation of the 'bound lipids is then to be regarded as breaking off the glycolipid (without amino acids) from the cell wall skeleton

A difficulty in the study of the activity of tuberculolipid fractions is their contamination with small numbers of intact bacillary bodies since it could possibly be maintained that the activity of any particular fraction is due to the high activity of such contaminants By the method of counting bacilli in stained smears small numbers of bacilli were found to be present in all the bacillary fractions and were estimated at about 10^4 bacilli per milligram or less than 01 per cent That these small numbers of contaminants could explain the adjuvant properties of chemical fractions is unlikely for the following reasons First the activity of wax D of *Myco tuberculosis* Canetti has been shown to be at least of the same order as that of heat killed intact bacilli Secondly the wax D fractions of the bovine type of *Myco tuberculosis* Marmorek and of *Myco smegmatis* and *Myco phlei* are ineffective though they too are similarly contaminated with trace amounts of intact bacillary bodies and it is shown above that bacilli from which these are derived are active

Recently A H E Marshall and I have tested the range of Dr Lederer s chemical fractions for their ability to promote lesions of allergic encephalitis in guinea pigs injected with homologous brain The latter was obtained aseptically from a healthy normal guinea pig and homogenized immediately in 1 per cent phenol saline This emulsion which included the dose of 16 mg weight of brain tissue was injected in Arlacel Bayol mixture into a hind foot pad The mycobacterial fractions (dose 1 mg) were added to the Bayol Animals were observed for periods of up to 30 days for the development of encephalitis When such symptoms appeared (12 to 2 days) they were usually followed by a rapid progression over 1 to 3 days to a moribund state The results are shown in Table IV alongside the results of the testing of the same fractions (in different groups of animals) for activity in increasing serum antibody levels and in producing corneal sensitization to ovalbumin The recorded numbers of animals with encephalitis included only those cases with severe neurological signs and in whom the presence of typical lesions was confirmed by histological examination

The results show that several different wax D fractions extracted from various human types of *Myco tuberculosis* proved active Successful promotion of encephalitis by purified wax and wax D fractions of *Myco tuberculosis* has been reported previously [21] although large doses were apparently required and some fractions were unaccountably inactive There are several reports [1, 8, 9] of failure with wax fractions We have

TABLE II. Effect of Mycobacteria and Fractions in Promoting Encephalitis with Homologous Brain and in Increasing Antibody and Corneal Hypersensitivity to Ovalbumin

Chemical Fraction	Reference Number	Number of Animals with Encephalitis (number of animals in group)	Average Serum Antiovalbumin (contemporaneous control figure in brackets) µg Ab N/ml	Corneal Responses to Ovalbumin (contemporaneous controls in brackets)
Heat killed *M. tuberculosis* human Canetti	WL9	4 (5)	509 (100)	3 3 3 1 (0 0 0 0 0)
Totally delipidated *M. tuberculosis* human Canetti	WL12	0 (5)	165 (149)	0 0 0 0 (1 0 1 0)
Neutral delipidated *M. tuberculosis* human H37Rv	WL31	3 ( )	394 (67)	3 3 3 3 2 (0 0 0 0 0)
Neutral delipidated *M. tuberculosis* bovine Marmorek	WL30	4 (5)	441 (67)	3 3 3 [illegible] 3 (0 0 0 0 0)
Wax D *M. tuberculosis* human Test	WL19	3 (5)	486 (86)	3 2 2 3 2 (0 0 0 0 0)
Wax D *M. tuberculosis* human Canetti	WL2	3 (5)	493 (100)	3 3 3 3 3 (0 1 0 1)
Wax C *M. tuberculosis* human Canetti	WL1	0 (5)	77 (158)	0 0 0 0 0 (0 0 0 1)
Purified cord factor *M. tuberculosis* human Brevannes	WL22	0 (5)	120 (67)	0 0 0 0 (0 0 1 0)
Wax D *M. tuberculosis* bovine B C G	WL7	0 (5)	91 (89)	0 0 0 0 1 (0 0 0 0)
Wax D *M. tuberculosis* bovine Marmorek	WL18	0 (5)	1[illegible]0 (10[illegible])	0 0 0 0 (0 0 0 0)
Wax D *M. tuberculosis* bovine Dupré S	WL26	0 (5)	241 (197)	0 1 0 0 1 (0 1 0 0 0)
Wax D *M. tuberculosis* avian	WL35	0 (5)	152 (107)	0 1 0 0 (0 0 0 0 0)
Wax D *M. phlei*	WL25	0 (5)	90 ([illegible])	0 0 0 0 1 (1 0 0 1 1)
Unextracted dried *M. phlei*	WL32	4 (5)	332 (124)	3 3 3 3 3 (0 0 0 1 1)

Eye reactions at 48 hours after intracorneal injection of ovalbumin. 1 = slight local opacity of cornea around site of needle puncture. 2 = moderate opacity of most of cornea. 3 = dense grayish white opacity and thickened cornea.

studied the wax obtained by extraction of heat killed human type *Myco tuberculosis* with isopropyl ether after previous treatment with acetone Wax prepared in this way has been reported to lack encephalitogenic action in guinea pigs[8] However chromatographic examination of the hydrolysate of this material gave no evidence of the presence of the above mentioned three amino acids

The close correspondence shown in Table IV between the activity of various whole and extracted bacilli and chemical fractions in promoting encephalitis and in increasing serum antibody levels suggests that the same chemical factor may be active in both instances Moreover these findings provide additional support for the hypothesis that the encephalitis is the result of an isoimmunization process in which the mycobacteria increase the immune response to an antigenic constituent of the brain tissue

The relationship of the cellular changes to this potentiation of the immune responses to ovalbumin and brain antigen is too involved a subject for full discussion here and now However, an outstanding finding with all chemical fractions which actively promoted encephalitis or antibody production to ovalbumin was the uniform presence of a widespread proliferation of macrophages many with epithelioid cell morphology In the control animals and those receiving inactive fractions the macrophage proliferation was considerably less and epithelioid cells were not seen The suggestion is strong that the epithelioid cell is essential for the adjuvant response While there is no evidence that these cells either contain or secrete antibody[21] they could possibly be concerned with the initial transfer or disposal of the injected antigen so that for instance its intracytoplasmic breakdown is delayed

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*Development of Arthritis in the Rat Following Injection with Adjuvant**

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The observation that a polyarthritis developed in rats following parenteral administration of an adjuvant mixture was reported from this laboratory in 1956[1,2] The original inoculum was a standard Freund type water-in-oil emulsion containing minced homologous skeletal muscle Subsequent study revealed that the muscle tissue was not a necessary component of the mixture and this was recorded briefly [4] It was noted that basic adjuvant when given in optimal dosage by the intradermal route had the ability to initiate following a suitable latent period variable degrees of joint and periarticular inflammation in a significant proportion of animals tested

Subsequent to the original observations multiple studies have been undertaken in an attempt to elucidate facts about the etiology and pathogenesis of these interesting lesions The investigations have followed three basic channels (*a*) further observations upon the incidence distribution clinical course and histopathology of the induced arthritis (*b*) attempts to isolate an infectious agent from the affected joint areas with particular reference to the pleuropneumonia like group of organisms and (*c*) efforts directed toward modification of the incidence and severity of the induced arthritic lesions This report will consider the results obtained in these experiments

MATERIALS AND METHODS

The subjects in these experiments have been both male and female adult rats of the Wistar and Long Evans strains Most animals have been bred on the premises but some have been purchased from commercial sources

This study from the Department of Medicine University of California School of Medicine and Wadsworth Hospital Veterans Administration Center Los Angeles has been supported by a grant from the Southern California Chapter of the Arthritis and Rheumatism Foundation and by Grant A 1286 National Institute of Arthritis and Metabolic Diseases National Institutes of Health U.S Public Health Service

After many observations it was concluded that females of the Long Evans variety gave the highest incidence of reproducible lesions and hence they have been used exclusively in the later experiments. The inoculum consisted of 4 parts thin mineral oil, 4 parts normal saline, 1 part emulsifier (Falba), and 1 mg dry weight of acid fast bacilli (*Mycobacterium phlei*) per milliliter of final mixture. Aqueous Merthiolate was added to a final dilution of 1:10,000 and the contents were emulsified in the usual way by the syringe technique. Bacteriologic studies on many samples have consistently been negative. Injections of adjuvant have been given intradermally into the skin of the posterior cervical region using a No. 21 needle and warmed inoculum (50° C). A total of 1.0 ml of adjuvant was deposited in several sites and it was found most satisfactory to enter the subcutaneous tissue and then re-enter from below the deeper layers of the epidermis before injecting the material. This technique avoids external leakage of the adjuvant. In some animals 0.5 ml of adjuvant was given on day 1 and another 0.5 ml on day 3. In order to evaluate more accurately the daily fluctuations in the arthritis a recording system was devised which was in some respects similar to the arthrogram used by Sabin and Warren[21] and others. It is illustrated in Figure 1. A

		LEFT						RIGHT			
FORE PAW	IP	4	3	2	1	BODY WT		1	2	3	4
	MCP										
		META CARPAL		WRIST		TAIL		WRIST		META CARPAL	
HIND PAW	IP	5	4	3	2	1	1	2	3	4	5
	MTP										
		META TARSAL		HEEL			HEEL		META TARSAL		

FIGURE 1 Arthrogram form

numerical grade of 0 to 4 was given for each joint of the four paws and tail, in which grade 4 represented the most severe involvement. The maximum score possible for any animal on any given day was 180 points.

Bacteriologic studies of inflamed tissues and blood cultures from affected animals were made on solid agar media consisting of a tryptic digest of beef heart fortified with human ascitic fluid as recommended by Dienes and Madoff[4] and a modified brucella broth* with added human ascitic fluid. The latter medium has proved the most satisfactory.

* Albimi Laboratories Inc., Brooklyn, New York

and details about it as well as more complete bacteriologic studies will be published subsequently

In other series adjuvant was prepared utilizing identical concentrations of different acid fast bacilli These included *Mycobacterium butyricum* a human nonpathogenic strain labeled F -1 * and an antigenic wax fraction of acid fast bacilli labeled Wax D Strain Canetti † Where killed phase I *Hemophilus pertussis* organisms were used in the adjuvant in place of acid fast bacilli the final inoculum contained $1\ 1 \times 10^{10}$ organisms per milliliter and 1 ml was given to each animal In experiments where *Streptococcus viridans* organisms viable or heat killed were used a concentrated saline suspension of 48 hour agar plate growth replaced the *Myco phlei* in the ratio of 0 3 ml of saline suspension (number of organisms undetermined) per milliliter of final adjuvant

In some series 200 r of total body radiation was given 24 hours before adjuvant injection Other animals were given two intracardiac injections 0 0 mg each of methylchlorethamine hydrochloride (nitrogen mustard) 48 and 4 hours prior to adjuvant inoculation To evaluate the effects of several antibiotics on the subsequent development of arthritis the following dosages were administered intramuscularly for 2 days before adjuvant and for at least 18 days thereafter (*a*) procaine penicillin 100 000 units once daily (*b*) streptomycin 1 5 mg twice daily (*c*) tetracycline, 6 5 mg twice daily

Results

Incidence

Close observation of animals in this laboratory with particular emphasis upon inspection during the tenth to seventeenth postinoculation days has revealed an overall occurrence of some degree of joint inflammation in 77 per cent of 280 animals given adjuvant alone It should be pointed out here that without such close scrutiny some of the mildest changes could easily be overlooked Hence transient swelling and redness of one or several small digital joints may appear on the eleventh or twelfth day in some animals and entirely disappear within 12 hours No such swellings were ever noted in uninjected controls In other animals similar initial swellings will have evolved into easily recognizable arthritis within the subsequent 24 to 48 hours In Figure is tabulated the percentage of affected animals in each of four arbitrarily estimated grades of severity It should be mentioned here that the induction of the arthritis in our

* Kindly provided by Mr E Taylor Peterson Department of Bacteriology and Serology Wadsworth Hospital Veterans Administration Center Los Angeles California

† Graciously donated by Dr E Lederer Institut de Biologie Physico Chimique, Paris France

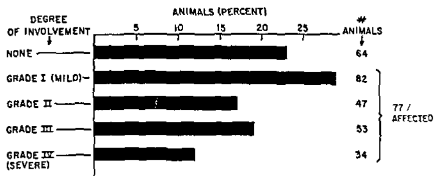


FIGURE 2 Incidence of arthritis in 280 animals compared with estimated severity

hands is still a variable and unpredictable procedure hence it has been necessary to utilize a control group of animals from the same source and age range with each of the test series designed to attempt to modify the incidence It was not possible to take a percentage incidence from the group as a whole and apply it at random to any individual study Although in many series of 10 to o animals arthritis developed in all or nearly all cases there was an occasional series in which the animals appeared to be absolutely or relatively refractory to the induction of arthritis

Effect of Site of Inoculation

As shown in Figure 3 the intradermal route of injection was by far the most satisfactory means of stimulating the development of arthritis No clear-cut explanation can be given for this difference unless one can be drawn from observations on the injection sites Immediately upon

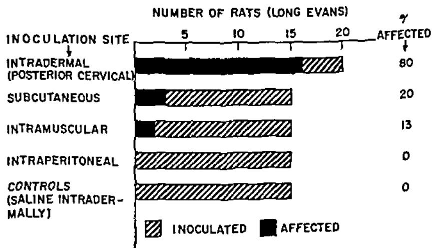


FIGURE 3 Incidence of clinical alteration in relation to the site of inoculation

injection rather large tense inoculum deposits can be seen and palpated within the skin In 24 hours the material has apparently dispersed somewhat and it is difficult to discern any abnormality By the fourth day a distinct nodularity can be observed at most injection sites and in the next 2 days these increase in size often measuring 1 by 1 5 cm On the seventh or eighth day the skin overlying some nodules becomes loosened and may slough exposing a red granulating lesion or a shallow purulent ulcer Most of these lesions must then become contaminated from their exposure but there has been no consistent correlation between injection sites that break down and the subsequent development of arthritis In about 30 per cent of animals there is no slough and the firm nodules remain intact for several months before finally resolving

Time of Onset

The latency period following injection of adjuvant has been one of the most constant features in this study In not a single animal has the initial sign of arthritis appeared before the tenth day and fully nine tenths of the rats that eventually develop lesions will do so between the eleventh and sixteenth days Occasionally the initial signs may be delayed for 3 or more weeks and in a very few animals to the fortieth day The discrepancy between the time of onset recorded in the present larger series and that averaging about 5 days mentioned in our initial series [1] is somewhat difficult to reconcile but the explanation may lie in the different strain of rats used in the two series or more likely in the fact that such a close and detailed daily inspection was not done in the initial series when we had not learned to recognize the minimal and very early lesions

Clinical Appearance and Distribution of the Arthritis

In all series the hindpaws were affected most severely and consistently Next in frequency came the forepaws and then the tail No clinical affection of the knee hip shoulder or other proximal joint could be recognized and this freedom from involvement was usually borne out by subsequent pathological examination

Acute Alterations The initial changes were usually recognized in one or both hindpaws At times a diffuse pinkish mild swelling of the ankle and dorsal aspect of the tarsus was observed In other animals focalized areas of swelling and redness were limited to the dorsum of a single or several metatarsal phalangeal joints or one or more proximal interphalangeal joints After the initial appearance of a lesion it may follow any one of three possible courses (*a*) the erythema and edema may rapidly subside within the subsequent 1 or 4 hours and leave no residual trace (*b*) it may remain unchanged for several days and then slowly resolve without

residuum occasionally to reflare again once or even twice, or (*c*) it may rapidly and progressively evolve into a full fledged and often intense *inflammatory response* The last is by far the most common course to be followed and a typical course in three moderately affected animals is shown in Figure 4 As is the usual case, the peak of severity is reached

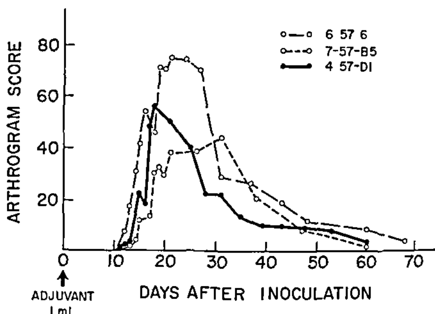


FIGURE 4 Typical time versus severity course of arthritis in 3 animals

on the eighteenth to twenty fifth days In the acute state the ankles and tarsal regions are most frequently affected then one or several of the toes the forepaws fingers and the tail Severely affected animals show swollen red paws and ankles that are increased in size two or threefold over normal They are obviously painful and tender to touch and serum exudes from the tense covering skin Such animals lie on their sides in obvious distress In no instance did an abscess form in a paw nor did draining sinuses develop Not uncommonly only a single or a few isolated joints were affected but these might show a grade 4 response and stand out in marked contrast to the adjacent normal appearing joints In the digits it was not rare for the lesions to present as fusiform swellings about the proximal interphalangeal regions (Figure 5) but diffuse involvement of one or several digits was more common The general appearance of the animals during the acute stages depended upon the degree and extent of the peripheral involvement Most animals appeared well but those with grade 4 involvement of several or all extremities lost weight, developed coarse ruffled fur, and generally were systemically affected The only

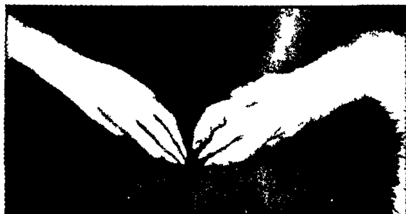


FIGURE 5 Multiple fusiform arthritis of digits and diffuse involvement of tarsus and ankle contrasted with a normal hindpaw

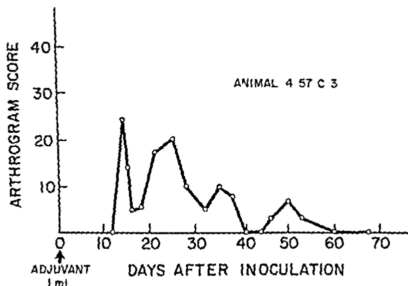


FIGURE 6 Undulating course of arthritis over a 2 month period

other clinical lesion noted was the presence of one or several 0 5 cm erythematous nodules in the external ears of three animals

Fluctuating Course The arthritis in one quarter of the animals followed a recurrent and fluctuant course often for several weeks following its initial appearance Shown in Figure 6 is the course followed by one such animal over an 8 week period This course graded as to intensity alone does not depict the entire picture since the arthritis in many of these

animals was migratory in nature affecting first some joints usually the smaller ones, and then others in a random fashion, leaving little or no evidence of residual affection in the recovered joints The arthritis in some joints underwent two or even three remissions and relapses It was only by careful daily charting that these oftentimes rapid transitions and fluctuations could be recognized with certainty Figure 7 portrays the migra-

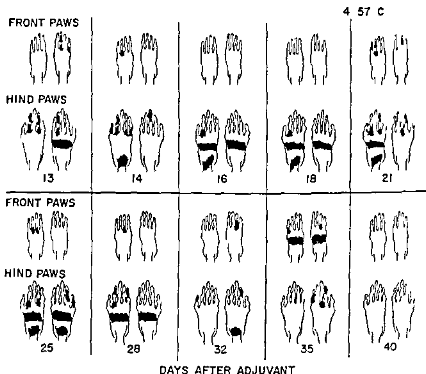


FIGURE 7 Graphic demonstration of the migratory nature of the polyarthritis in one animal

tory nature of the polyarthritis in one of the animals Rarely did the exacerbations appear after 2 months although in two animals minor recurrences were observed at the fifth and sixth month respectively

Chronic Alterations Most of the animals that manifested grade 4 acute arthritis had some lasting evidence of their disease especially if the acute condition affected the heel wrist, or metatarsal phalangeal joints In these cases it was not clear from the clinical viewpoint whether there was some residual smoldering inflammation or whether the alterations were the end result of fibrosis due to extreme inflammatory reaction It was certain however that the lesions in some joints labeled as grade 4 in the acute stage could recover completely without residuum The chronic changes took the form of firm enlargements especially about the ankle and less commonly

at the metatarsal phalangeal joints and in the tail Ankylosis often partially or completely restricted motion of the ankles and subluxations with flexion deformities produced a flipper hand on occasion In a few animals with severe acute arthritis of the ankles there was residual permanent fixation in extension

Roentgenologic Alterations

In some of the animals with more extensive acute involvement evidence of bone and joint change was noted about 4 weeks after the acute onset During the chronic or recovery phases up to 4 months the x ray showed the progressive development of a periostitis followed by nodular and feathery exostoses along the entire shaft or an isolated segment of bone These changes were most marked about the os calcis and ankle joint (Figure 8)

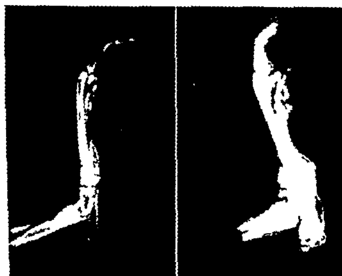


FIGURE 8 X ray of normal hindpaw on the left contrasted with chronic (3 month) arthritic paw Note multiple exostoses about the os calcis and metatarsal heads

but were seen on occasion also on the lateral wings of the caudal vertebrae The ankle joint the only joint acutely affected that was large enough to visualize accurately on the x ray film showed some narrowing to the point of complete obliteration in a few instances

Pathologic Observations

Gross examination of the inflamed paws in the acute stages disclosed a swollen edematous hyperemic tissue In the subacute and chronic phases a rather dry firm proliferative tissue enveloped the plantar and dorsal

tendons etc No loculations of fluid or abscesses were encountered, and so far as could be determined there was no distention of the synovial spaces by fluid accumulations The exposed joint surfaces appeared normal except in the chronic situation where fibrous ankyloses were found and the articular surfaces were adherent completely or at their margins

Histologically in both the acute and chronic periods the most striking feature was an activation of the connective tissue elements In the earliest lesions there was edema with moderate inflammatory cell response In lesions of a few days duration fibroblastic and histiocytic proliferation predominated with the active fibroplasia localized to a large extent in the immediate paraarticular and paratendonous regions (Figure 9) Variable but not excessive numbers of acute and chronic inflammatory cells were present in the involved areas The lining cells of the synovial sheaths of the tendons and joints adjacent to the involved connective tissues were prominent and often reduplicated The joint spaces were invariably free of inflammatory exudate except for an occasional leukocyte Vascular granulation tissue on occasion was observed growing over the lateral margins of the articular surfaces from the adjacent synovial membrane Similar tissue encroached upon the subchondral bone the articular cartilage and even into the cortical shaft in some animals (Figure 10) Included in the connective tissue response was the other striking feature an excessive but focalized activation of osteoblasts This was largely confined to the paraarticular zones but occasionally presented as segmental areas of proliferative activity along the shafts Osteoblasts and osteoclasts were numerous not only on the periosteal surfaces adjacent to granulation tissue but also about the bone spicules in the marrow Such a response was segmental even in the shaft of a single bone

In the subacute stages the connective tissue overgrowth was more apparent in quantity Inflammatory reaction was less impressive The most significant changes were granulomatous and fibrous pannus covering portions or all of the articular cartilages of some joints but sparing most others and deposition of layers of new bone along the cortical shafts There were focal areas of edematous connective tissue present in some regions and occasionally these contained strands or clumps of hyaline pink material resembling fibrinoid

In the chronic stage usually 4 to 6 months following the acute lesions the previously hyperplastic connective tissue was dense contracted and had numerous but not prominent fibroblastic elements Adjacent articular surfaces were adherent completely or at their margins by the fibrous connective tissue (Figure 11) Some joints were fused by bony ankyloses The new periosteal bone was organized and thickened into broad trabeculae The cortical and medullary bone occasionally gave evidence of some reorganization with broadened spicules etc Even at this late date

FIGURE 9 Connective tissue and synovial proliferation at the margin of a tarsal joint with marginal extension into the joint space H & E ×80

FIGURE 10 Marked paracapsular fibroplasia with granulation tissue excavation of the cortical bone in two places (arrows) and erosion of a portion of articular cartilage by activated connective tissue elements (? chondrocytes) H & E ×120

FIGURE Chron c fibrous ank lo s of a tarsal o nt at 4 n onths Note bon exostoses th formed marro ca t es o erl ng the or ginal cort cal surface H & E ×80

FIGURE 12 Small sarco d l ke ep thel o d granuloma n the l ver ad acent to the central ve n H & E ×270

some foci of activity persisted in areas. Hence edematous nodules in connective tissue contained strands of fibrinoid like material and were surrounded by proliferating fibroblastic and histiocytic elements and a few mononuclear cells.

Except for the changes described in the clinically apparent locations the other joints and connective tissues were uninvolved with the exception of a modified but similar finding about the knee joints in a few instances There were rare low grade nonnecrosing perivascular infiltrates in the regions of the involved tissues only The nodules noted in the external ears of a few animals proved to be focal mononuclear cell collections in rather edematous connective tissue adjacent to the auricular cartilage

Detailed examination of the viscera has been negative except for two interesting lesions The first was a small focal granulomatous sarcoid like nodule in the liver parenchyma (Figure 1) seen in about o per cent of the animals but likely to have been found in many more if a thorough search had been made for them They contained no accompanying inflammatory cells and the essential element was a prominent connective tissue or histiocytic (epithelioid) cell Also found in the liver was an occasional necrotic or organizing focus containing some proliferating connective tissue The second curious lesion was a nodular granulomatous reaction in the lungs This was observed in varying degrees in fully three fourths of the adjuvant injected animals irrespective of the presence of peripheral joint pathology and had greatest predilection for the subpleural zones Occasionally it assumed significant proportions and displaced more than 30 per cent of the functional pulmonary tissue Histologically it was a noncaseating nodule whose main cellular component was a macrophage often demonstrating epithelioid cell characteristics (Figure 13) Giant cells of the Langhans type were common These lesions resembled in many ways other forms of granulomatous pneumonitis including sarcoid

Bacteriologic Studies

Moist tissue smears and loop cultures from inflamed periarticular tissues have been studied repeatedly on the usual aerobic and anaerobic solid agar media without isolation of a consistent bacterial organism Occasional growth of a rapidly spreading *Proteus vulgaris* was thought to be due to contamination since similar sporadic growth was obtained from control tissues

Extensive efforts continue to be made to define the role if any that the pleuropneumonia like organisms (PPLO) might play in the etiology of these lesions Details of this phase of the investigation will be presented in a subsequent publication and will only be summarized here In the inflamed periarticular tissues from 6 of 11 severely affected animals PPLO

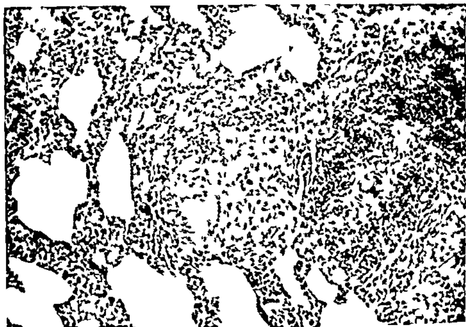


FIGURE 13 Noncaseating sarcoid like pulmonary granulomas Note epithelioid and Langhans type giant cells H & E ×135

have been cultured in the broth media as outlined briefly before In none of the animals with more mild lesions have positive cultures been obtained At times it was necessary to incubate anaerobically for 2 to 4 weeks before satisfactory evidence of growth appeared The cultural characteristics and colony morphology of the various PPLO s were extremely variable when observed on solid media transplants From at least one animal two strains were separable by antibiotic sensitivity and serologic testings There was also considerable variability in the antibiotic sensitivities of the other PPLO – so much so in fact that it was quite clear we were dealing with multiple strains of PPLO Negative cultures have been obtained from several less severely affected animals and from controls but this matter has not been pursued completely as yet

Centrifuged broth concentrates of heavy growth of various strains of viable PPLO as well as saline suspensions of solid agar scrapings have been given in amounts up to 1 5 cc intracardiac in many rats in an attempt to induce an arthritis in the absence of adjuvant In no instance has any evidence of illness developed in these animals aside from an initial post injection listlessness which lasted at the most for 18 hours

In a number of animals a saline suspension of viable PPLO was given subcutaneously into the dorsum of one hindpaw in control animals and to another group which at the same time received an adjuvant injection by

the usual intradermal manner In the large majority of both groups the injected paw returned to normal after a few hours of localized edema In an occasional animal in either series a small reddish nodule formed at the inoculation site by the third day Aspiration in some instances revealed purulent material from which PPLO could be cultured There was no apparent effect of the paw injection on the incidence of arthritis or its subsequent systemic or localized development in the adjuvant injected animals Injection of a saline suspension of PPLO into the knee or ankle joint did not elicit any inflammatory response

Attempts to Modify the Incidence and Severity of the Arthritis

Other Mycobacteria Myco butyricum and a nonpathogenic acid fast bacillus (F 1) were substituted for *Myco phlei* in two adjuvant mixtures An antigenic wax component of acid fast bacilli was supplanted in another series and in a fourth an adjuvant of differing basic constituents but including *Myco butyricum** was tested As is shown in Figure 14 all of the preparations were effective in inducing an arthritis From the small numbers of animals used in each series no conclusions could be reached as to the relative potency of each adjuvant but the impression

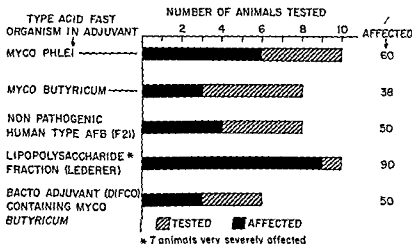


FIGURE 14 Effect of different mycobacteria and their components on induction of arthritis

Bacto Adjuvant Incomplete (Control #430413) kindly supplied by the Difco Laboratories Inc Detroit, Michigan This material was fortified with *Myco butyricum* (#431268 Difco) to bring its final concentration to 1 mg/ml

was gained that the wax fraction induced a more severe illness in a greater proportion of animals tested

Substitution of Other Organisms for Mycobacteria Three groups of 6 animals were given intradermal injections of oil emulsion containing in place of mycobacteria suspensions of killed *H pertussis*, or viable or heat killed *S viridans* organisms in concentrations as outlined previously In no instance did an arthritis develop nor did any of the animals appear to be adversely affected by the inoculum The typical granulomatous nodules as seen at the site of inoculation in mycobacteria fortified adjuvant did not develop in any of these animals

The Effect of Adrenalectomy, ACTH, or Cortisone In two series of animals a bilateral adrenalectomy was performed The animals were maintained thereafter on 1 per cent sodium chloride orally, and following recovery one week later adjuvant was injected Arthritis appeared initially on the eleventh postinoculation day and a total of 13 of 18 animals or 76 per cent were affected There was no question that in general the arthritis was much more severe in all these animals than it was in the controls Arthrogram records were not kept at the time these experiments were conducted so quantitative values cannot be given

ACTH in a dose of 3 mg per kilogram of body weight per day was given to another group of rats prior to and for 3 weeks following adjuvant injection Seven of 1 animals so treated developed arthritis of a moderate degree

Cortisone acetate was likewise given parenterally to an additional 6 rats in a daily total of 4 mg per kilogram In the small series studied, 2 of 6 animals developed a slight but definite arthritis It was quite clear that cortisone did not enhance the frequency or intensity of the arthritis in this small series

The Results of Several Stressful Procedures Applied in Conjunction with Adjuvant As depicted in Figure 15 oo r of whole body irradiation given 4 hours prior to adjuvant injection had no enhancing or depressant effect on the subsequent development of arthritis when compared with the control nonirradiated group In contrast nitrogen mustard administered parenterally 48 and 4 hours prior to adjuvant seemed to have a potentiating effect upon the severity of the arthritis but final decision on this point must await further confirmation There were 10 animals in each of these three series and 8 8 and 9 animals developed arthritis in the control the irradiated and the nitrogen mustard treated groups respectively

An attempt was made to observe the effects of large daily doses of parenteral cortisone (5 mg per animal) on the development of arthritis

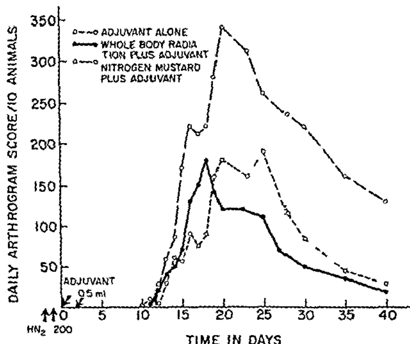


FIGURE 15 Effect of body radiation or nitrogen mustard on severity of arthritis

The mortality rate in these animals was extremely high most dying in 3 to 6 days of confluent pneumonia and other evidence of sepsis In none of these animals was there any evidence of arthritis Of the 3 animals that survived this massive daily cortisone dosage for 16 days all had lost considerable weight and one developed arthritis of a mild to moderate degree

The Effects of Adjuvant Plus PPLO Intracardiac 48 hour broth culture and saline suspensions of a PPLO (strain X28) isolated from the periarticular tissues of one of our rats was given in 1.5 ml quantities at various intervals after adjuvant injection As shown in Figure 16 when PPLO was given 1 4 or 9 days after adjuvant there was no significant variation in incidence or severity as compared with controls (not shown in this figure) However when the organisms were given on the seventh day there was a significant twofold potentiation of the severity and a prolongation of the duration of the arthritis The incidence in both groups was about the same in that 8 of 10 controls receiving adjuvant alone showed symptoms compared with 9 of 10 given supplementary PPLO on the seventh day This experiment was repeated in part again using controls and 7 day PPLO injections and the potentiating effect of the latter was even more striking on this occasion

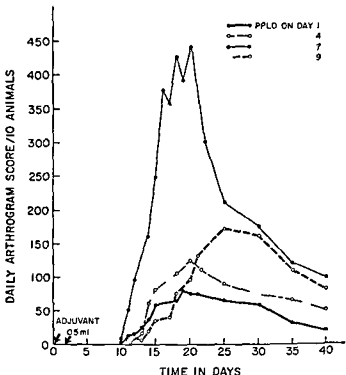


FIGURE 16 Enhanced intensity of arthritis by seventh day intracardiac injection of pleuropneumonia like organisms

EFFECT OF VARIOUS ANTIBIOTICS ON ADJUVANT INDUCED ARTHRITIS

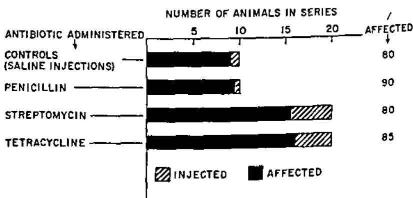


FIGURE 17 Effect of various antibiotics on adjuvant induced arthritis

Trials with Various Antibiotics and Adjuvant Three series of animals were given daily injections of various antibiotics for 48 hours before and during the entire latency period after adjuvant inoculation The antibiotics were continued for several days after appearance of the arthritis As recorded in Figure 17 the antibiotics in the relatively high dosages selected for trial had no effect on the incidence or in fact on the severity of the adjuvant induced arthritis The streptomycin and tetracycline series were done a second time since these agents especially the latter have been reported to have an almost universal inhibitory effect upon the *in vitro* and *in vivo* PPLO growth The results in the second series duplicated the first

COMMENTS

The central theme of this investigation revolves around an inquiry into the etiology of this interesting but perplexing rodent arthritis The double question that seemed most pressing was Is this lesion the result of a hypersensitivity response to an acid fast fraction in the adjuvant or does it arise secondarily to the activation of a latent infectious agent in the animal? The results obtained in this study do not conclusively answer this question but they do provide a reasonable measure of evidence in favor of the hypersensitivity element

The original premise taken was that the arthritis resulted from an allergic reaction This was rapidly modified following discussions with some of my colleagues who suggested that a pleuropneumonia like organism could be the dominant factor This suggestion was not unreasonable since a form of arthritis in rodents has been associated with PPLO on many occasions [3, 6] in both the natural setting and the laboratory Furthermore this same type of infectious agent has been cultured from purulent arthritis and tendovaginitis in cattle sheep goats pigs chickens and even very rarely in man [5, 12] Using arthrotropic strains of PPLO a poly arthritis has been readily reproduced in rats and mice [18, 21] inhibited by streptomycin and the tetracyclines and even utilized as a model to test various antiarthritic agents [17] Three significant points should be made about this infectious arthritis which are at variance with the lesions observed in adjuvant treated rats (1) The PPLO arthritis usually appears within 4 to 96 hours following the injection of the infectious material (2) The lesions are those of a purulent infection with an actual suppurative joint cavity invasion in contrast to the proliferative connective tissue response of the adjuvant arthritis which only secondarily invades the joint with granulomatous pannus An exception seems to be the lesions in mice as described by Sabin and Warren [21] (3) The inhibition of the infectious arthritis by the broad spectrum antibiotics contrasts with the complete resistance of the adjuvant induced arthritis to these agents

Why is it then that PPLO in pure culture can be isolated from the inflamed tissues What relation does their presence have to the development and evolution of this arthritis The second question cannot be answered with any degree of certainty at the present time and the only comment that can be made is that each organism isolated appears to be distinctive from the others by serologic and antibiotic sensitivity testings with some overlap It would be most unusual to have several distinct strains reproduce an identical pattern of disease In an attempt to explain the presence of these organisms in the tissues it should be pointed out that unique and sensitive cultural techniques plus lengthy incubation periods have been utilized In some instances where paired cultures were made on standard PPLO media growth rarely developed on the latter With this fact in mind perhaps it is justifiable to wonder whether such organisms might not be frequent nonpathogenic symbionts in rodent and other animal tissues Perhaps under conditions of tissue inflammation they are stimulated to proliferate and may even contribute something to the tissue lesion itself Perhaps pertinent here are some recent observations by Schaedler and Dubos who noted that chronic bacterial infections of several types could be activated by suspensions of killed mycobacteria or pertussis organisms PPLO and L forms are common inhabitants of the upper respiratory tract of rodents and of the female genital tract of humans and are found in many other situations These ubiquitous agents have recently been discovered in many unexpected places including sterile tissue cultures of HeLa cells in routine laboratory use embryonated chick egg and apparently normal rodent lung and brain tissue [21]

Other organisms that have been associated with an arthritis in man and animals but need not be considered here include the usual pyogenic bacteria *Streptobacillus moniliformis* an *Erysipelothrix* organism [8] a microorganism resembling *Nocardia asteroides* and perhaps representing its L form [9] various viruses when administered intraarticularly [3] as well as some bacterial extracts [6]

The case is somewhat more impressive for hypersensitivity as the pathogenetic mechanism whereby these lesions are produced But hypersensitivity to what The most potent antigens administered to the animals are of course contained in the mycobacteria and it is here that we turn for an answer Since the first observations of Lewis and Loomis [11] that virulent living tubercle bacilli injected intraperitoneally into guinea pigs could enhance antibody formation to various antigens given by the same route there has been an ever increasing amount of confirmatory evidence to support this effect of the acid fast organism Raffel and coworkers [19] have attributed such an effect to a certain lipopolysaccharide or wax fraction of the organism Many workers [7, 15] feel that this fraction is not itself an antigen but serves as a sensitizer to render anti

genic any appropriate material incorporated into the inoculum Circulating antibody has not been found following its injection[5] White Coons and Connolly [3] have undertaken a study of the histologic response to injections of a purified wax fraction incorporated into a water in oil emulsion in guinea pigs Their descriptions of the clinical and histologic events that transpire in an injected guinea pig paw duplicate completely the sequence observed in the local injection sites in the posterior cervical region in the rat Furthermore they have observed small granulomata in the liver and a striking gross and histologic picture of granulomatous pneumonitis in the lungs of all animals These responses are identical with those found in our rats In a series of shrewd observations to be elaborated upon in this symposium Chase Slizys and Allen have described a disseminated granulomatous disease in guinea pigs which follows the second deposit of acid fast material into an animal after a properly spaced interval I have had an opportunity to study the histology of some of these lesions and find among other things that the histiocytic and connective tissue proliferation in the subcutaneous location resembles the reaction in the periarticular areas in the rat In addition the small granulomas Chase *et al* find in the lungs and liver are identical with those observed in the rodent *

Here then are two experimental examples of closely similar or identical histologic events which transpire following the administration of acid fast material to another animal Chase s guinea pigs did not develop arthritis but did on occasion manifest transient swellings of the legs and at times nodules occurring above the wrist joint or involving the joint of a digit

In the rat several examples of adjuvant induced arthritis have been observed but most are unpublished In 1954 Stoerk Bielinski and Budzilovich[24] described in an abstract the development of an arthritis identical with that described herein in about one half of 86 rats that received mycobacteria containing adjuvant in which a homogenate of homologous or heterologous spleen cells was emulsified They observed the lack of influence of various antibiotics on the appearance of arthritis and were unable to culture PPLO from several tissues including the joints Their conclusion was that the splenic antigen had something in common with the synovia and the arthritis was a result In several laboratories throughout the country the adjuvant induced arthritis has now been produced with varying degrees of success Dr Waksman has seen it appear in some of his rats that also developed an allergic encephalomyelitis to brain antigen but apparently there was no correlation between the incidence of the two phenomena

* It cannot be concluded *a priori* at this time however that both the peripheral and visceral lesions in the rat have the same etiology

In the original paper[13] it was pointed out that deletion of the mycobacteria (or likely also the wax fraction) from the oil phase of the adjuvant prevented the development of the arthritis as did the injection of the acid fast organisms in the aqueous phase alone Presuming then that the arthritic lesions develop as a result of some response by the rat to a product of the acid fast bacillus can this be considered to be a hypersensitivity response? If so to what One of the strongest arguments in favor of hypersensitivity is the invariable interval of at least 10 days before appearance of the initial lesions Since similar or even more intense lesions were induced in animals given Lederer s wax fraction in the water in oil emulsion * it would seem that the lipopolysaccharide element may be implicated in the pathogenesis of the arthritis Some support for this as outlined before, comes from the work of White, Coons and Connolly[25] with the wax fraction alone

The findings that whole body radiation or nitrogen mustard administered prior to adjuvant had little effect upon the subsequent development or intensity of the arthritis is a little disturbing if one is to consider that this lesion is the result of a hypersensitivity reaction of the conventional type These experiments have not however been of an all exclusive nature The unequivocal potentiating effect of intravenous PPLO when given only on the seventh postadjuvant day might be attributed to a non specific stress factor (accelerator) administered at a critical stage in the evolution of the underlying phenomena This matter should be investigated further

As mentioned before, most workers in the field consider the lipopolysaccharide fraction to be of nonantigenic character Freund[7] believes that the function of the wax fraction in adjuvant is to incite a cellular reaction on the part of the inoculated host Most of the cells that respond to the injection are not of the inflammatory type but rather are epithelioid cells which basically are of mesenchymal tissue origin Certainly the most prominent histopathologic feature of the arthritic lesions is an overwhelming stimulation of all of the connective tissue elements (fibroblasts histocytes osteoblasts synoviocytes etc) in the affected areas With these facts in mind the following queries could be posed Is it possible that the observed reactions are due to an intense focal stimulation of the connective tissue elements by the wax fraction of the mycobacteria perhaps through temporary alteration of cell enzymes Why then the inevitable incubation period before the appearance of the arthritic process Is it likely that the injected adjuvant may incorporate some of the host tissues into it after injection render them antigenic and subsequently induce an autoantibody response directed particularly against the connective tissues in the para

* The concentration of lipopolysaccharide in wax adjuvant was considerably greater than in the mycobacteria fortified adjuvant

accepted as characterizing allergic reactions that is reactions based on immunologic events They are produced by specific immunization of experimental animals A latent period intervenes between immunization and the appearance of lesions it may be as short as 8 or 9 days and is clearly related to the dose of immunizing antigen After a cycle of lesion formation there may be a remission of the disease process re exposure to antigen in the proper manner at this time results in reappearance of disease after a shortened latent period an anamnestic response (Condie [19]) Immunologic specificity is beautifully illustrated by the elicitation of the inflammatory response only in the tissue corresponding to that used for immunization even central and peripheral nervous system myelin are distinguished though here characteristic immunologic cross reactions may occur [5] The reaction as described in the foregoing paragraphs appears always to be fundamentally the same no matter in which tissue it is elicited the perivenous collection of lymphocytes and histiocytes with invasion and destruction of the antigen containing parenchyma While passive transfer of humoral antibody does not reproduce the lesion in normal recipients a moderately successful passive transfer of allergic encephalomyelitis with living sensitized lymphoid cells has been achieved [1] Desensitization is not successful but a type of immunologic unresponsiveness may be produced by single injections of antigen in the newborn animal or repeated injections in the adult with loss of the ability to develop disease [2, 6, 7, 20, 25]

The available evidence justifies the identification of the autoallergic diseases as hypersensitive reactions of the delayed or tuberculin type The importance of mycobacteria and the intradermal route of inoculation in the technique of sensitization the nature of the histologic reaction itself the failure of passive transfer with serum and the partial success with living lymphoid cells and finally the susceptibility of the disease process to ACTH and cortisone therapy are all features characteristic of delayed reactions as distinct from cytotoxic anaphylactic or Arthus reactions which are associated with circulating antibody In several autoallergies delayed skin reactions to homologous tissue have been demonstrated which show a partial correlation in time and degree with the disease process and in allergic encephalomyelitis corneal reactions to homologous tissue have been found as well

The Meaning of Autoantigenicity

The terms autosensitization and autoimmunization have been applied not only to the autoallergic diseases under discussion which as we have seen are inflammatory processes probably of the delayed hyper sensitive type but also to a group of human diseases in particular acquired

hemolytic anemia certain chronic leukopenias idiopathic thrombocytopenic purpura paroxysmal cold hemoglobinuria and disseminated lupus erythematosus in which serum factors are found which have serologic properties like those of antibody (reviewed in reference 50) These serum factors appear responsible for some at least of the manifestations of these diseases While other evidence permitting their identification as autoantibodies is lacking their *in vitro* and *in vivo* behavior can be duplicated experimentally with the use of heteroantisera Autoantibodies are found in additional human diseases among them viral hepatitis and other liver disease glomerulonephritis and other renal disease myocardial infarction rheumatic fever rheumatoid arthritis other collagen diseases and syphilis the antigens being found in liver, spleen kidney heart muscle joint etc Here there is not the slightest evidence that the antibodies if such they are play a pathogenetic role and except in the case of glomerulonephritis the use of heteroantiserum experimentally fails to produce a lesion resembling that in man Rather it would appear probable that they are formed in response to tissue breakdown in the various disease processes The autoantibodies found in multiple sclerosis chronic thyroiditis sympathetic ophthalmia and chronic adrenal disease I believe to belong in this category as well

The distinction between the three types of autosensitization considered in the previous paragraph may be accounted for tentatively on the basis of the remoteness of the antigens concerned from the blood stream and consequently from the immune apparatus Tissue antigens which reach the blood stream in negligible quantities if at all whether because of the special nature of their metabolism or because of blood tissue barriers may be regarded by the immune apparatus as foreign substances They can then induce antibody formation or delayed reactivity the latter type of response may result in inflammatory (hypersensitive) reactions to antigen wherever it is present in the tissues that is in autoallergic disease Other tissue antigens less remote from the circulation may be unable to produce delayed sensitivity perhaps because enough antigen is released from time to time to induce a state of partial immunologic nonreactivity like those discussed elsewhere in the present symposium These antigens can however still elicit the formation of circulating autoantibody when as a result of some destructive process affecting tissue containing the antigen quantities sufficient to immunize are released The antibody in turn can cause disease by its direct effect on antigen containing cells when these are readily accessible as in the case of the blood elements and vascular endothelium More often it fails to produce any lesion at all In the case of glomerulonephritis in which a readily accessible noncellular material is the antigen the lesion produced may be of the Arthus type Finally antigens like serum proteins which are present in the circulation in high

concentration at all times may produce a higher order of immunologic nonreactivity perhaps similar to Felton's immunologic paralysis and are unable to act as autoantigens at all Actually the antigens implicated in the experimental autoallergies are separated from the circulation by striking barriers in many cases the blood brain barrier blood aqueous barrier basement membrane of the seminiferous tubule and acinar epithelium of the thyroid follicle being examples The evidence that autoantigenicity may depend on failure to develop tolerance to an antigen during fetal life because the antigen is absent at the appropriate time is contradicted by the fact that many autoantigens actually are present during fetal life and by evidence that repeated exposure to antigen of adult guinea pigs rabbits or rats depresses these animals ability to develop allergic encephalomyelitis[a] or phaco anaphylactic endophthalmitis

The Relation of the Experimental Autoallergies to Human Disease

The experimental diseases under consideration tend to have a prolonged remittent course since activity of the disease process is determined by the simultaneous presence of antigen normally found in an affected tissue and of sensitivity of the appropriate type above some threshold level The human diseases which resemble the experimental autoallergies (Table I) fall into two distinct groups those in which a single episode with a sharply self limited course occurs and those characterized by a prolonged course with or without clearly distinguishable remissions and exacerbations The latter category includes two cases in which autoimmunization is almost certainly occurring the diseases affecting the lens (phaco anaphylactic endophthalmitis) and the uvea (sympathetic ophthalmia) In both of these groups a traumatic release of tissue antigen provides the immunization which after a suitable interval results in the production of disease this in turn has been shown to be rather well correlated with delayed skin sensitivity to tissue antigen In both a prolonged or remittent course is characteristic A disease picture indistinguishable from that of acute multiple sclerosis may be produced in man by rabies vaccination that is immunization with nervous tissue[2] In chronic polyneuritis chronic thyroiditis cytotoxic contraction of the adrenal and chronic infertility a prolonged or remittent course is seen but without clear evidence for an original immunization with tissue antigen or for the presence of sensitivity Presumably some or all of these must also be due to autosensitization

The group of human diseases which have a self limited course includes encephalomyelitis polyneuritis and uveitis all customarily regarded as

post- or para infectious sequelae of viral infections (chiefly vaccinia measles varicella and rubella), and orchitis normally considered as part of the mumps infection Thyroiditis ovaritis and pancreatitis are less frequent postinfectious sequelae of the same type The self limited character of these diseases their usual association with viral infections especially the exanthems their failure to recur all are features which suggest the interpretation of these diseases as hypersensitive reactions to virus rather than to tissue antigens It is well to recall that von Pirquet[45, 49] accumulated substantial evidence that the rash in smallpox and measles was probably a hypersensitive reaction to widely disseminated virus More recently it has been demonstrated that delayed sensitivity to mumps virus is present in the infected patient at the time the symptoms of mumps parotitis first appear[46] Is it not reasonable to suppose that the same hypersensitive response which produces an inflammatory lesion wherever virus is present in for example the skin must also produce one wherever virus is present in the nervous system or other tissues? In favor of this concept is the fact that the interval between viral disease and postinfectious complication is usually too brief for autosensitization to occur The onset of the apparent autoallergy occurs within a few days of the onset of viral disease often simultaneously with it, or indeed in some cases may precede it by a day or two[43] Finley[36] has shown that the onset of post vaccinal encephalomyelitis corresponds very closely to the day of maximum reaction at the vaccinated site, von Pirquet's acme *It should be remarked* that phenomena which we have usually been willing to associate with the viral infection as such do not all occur at the same time Fever and the involvement of mucous membranes precede the appearance of the rash by one or more days The rash itself first appears in different areas of the skin on different days Similarly postinfectious neuritis in general starts later than postinfectious encephalomyelitis[43] The relative infrequency of the postinfectious sequelae is not inconsistent with the picture drawn here We do not call generalized vaccinia an autoallergy of the skin because it is uncommon, nor has mumps orchitis which occurs in perhaps one out of five cases of mumps been regarded as nonviral for this reason The histological similarity of the lesions in the postinfectious sequelae to those *in the autoallergic diseases* must be attributed to their being hypersensitive reactions of the same type in the one case to virus and in the other to tissue antigen

Summary

The experimental autoallergic diseases are reproducible experimental entities which appear to constitute a well defined group with constant histologic and other characteristics They provide excellent experimental

models for para infectious diseases in man which are interpreted as hypersensitive reactions to virus and for certain chronic remittent human diseases of unknown origin some of which have been demonstrated to have an autoallergic mechanism

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DESIGNATED DISCUSSION

LEON SOKOLOFF (Bethesda, Maryland) Our laboratory * is concerned with experimental joint disease and we have followed Dr Pearson s studies with much interest It is generally recognized that a major stumbling block in approaching the pathogenesis of rheumatoid arthritis and similar disorders is the lack of a suitable experimental model and that research in this area would be greatly facilitated if a reproducible method for induc ing a self procreating noninfectious, inflammatory joint disease were available

My associate Dr Emanuel Silverstein has repeated some of Dr Pear son s and Dr Stoerl s experiments and has obtained results that confirm their findings in a large part We would however entertain interpreta tions of the lesions different from those presented in two respects

The experiments are summarized briefly in Table I

TABLE I ARTHRITIS EXPERIMENTS USING FREUNDS ADJUVANT DIFCO MYCOBACTERIUM BUTYRICUM

Exper iment	Animal			Inoculum				Incidence† Arthritis
	Type	Age	Sex	Type	Dose (ml)	Conc * Myc	Site	
1	Rats (2 3 5)	8–16 mos	M	Spleen	0 2	1	s c neck	0/17
2A	Rats (1 3–7)	8–19 mos	M F	Muscle	0 1	4	s c paw	11/49
2B	Rats (1 7)	8–19 mos	M F	Spleen	0 4	1	s c back	6/35
3A	Rats (8)	2 mos	F	Muscle	0 2 0 4	5	i c paw i c back	0/10
3B	Guinea pigs	5–6 wks	F	Muscle	0 2 0 4	5	i c paw i c back	0/10

Rat strain
1 Albany
2 AxC 9935
3 Buffalo
4 CW R
5 Fischer
6 M520
7 OM/N
8 Sprague Dawley

s c subcutaneous
i c intracutaneous

Conc myc mg mycobacteria ml inoculum
† Incidence arthritis number of rats with gross involvement of paw/number of inocu lated rats

Sterile mixtures of rat muscle or spleen and Freund s adjuvant with *Mycobacterium butyricum* were employed They were injected by either the subcutaneous or intracutaneous route in the several sites noted in the chart Large amounts of mycobacteria 1 to 5 mg per milliliter were

* National Institute of Arthritis and Metabolic Diseases National Institutes of Public Health U.S Public Health Service

present in the inoculum The experimental animals comprised 8 strains of rats as well as a group of guinea pigs In the last column it is seen that the frequency of the lesion varied greatly from experiment to experiment but in group A 11 of 49 injected rats developed gross swelling or redness of the paws and in group B 6 of 35 The other experiments were completely unsuccessful

Histological examination of the affected extremities was carried out in only 5 animals during the acute or active stages of the inflammation in the remainder it was made after the inflammation had burned out The microscopic appearance in these paws by and large corresponds to that described by Dr Pearson, but in addition 3 of the 5 had another component that we would regard as granulomatous (Figure 1)



FIGURE 1 Subcutaneous inflammatory reaction and periosteal new bone formation in the left tarsal region following injection of adjuvant into right paw The joint is spared

This figure is a sagittal section through an inflamed left tarsal region of a rat that had been injected with muscle and adjuvant in the right hind paw From above down we observe skin subcutaneous tissue heavily infiltrated with inflammatory cells then a thick zone of coarse periosteal new bone formation and finally the dorsal portion of the calcaneotarsal joint Your attention is directed to the cellular infiltrate there is a diffuse

inflammatory reaction that is best regarded as a cellulitis In add however there are two pale miliary nodules of a different character

Under higher magnification (Figure) the nuclei of the cells ma up the nodules are seen to be elongated and usually somewhat curved cytoplasm is pale cell outlines are poorly delimited and at tim s ap ently syncytial The cells frequently have a whorled arrangement appear to be epithelioid cells In other nodules not illustrated here isola multinucleated giant cells or large round vacuoles were present

We would interpret these lesions as granulomata comparable to th seen at the site of injection of the adjuvant and in the viscera Sim lesions were present in the subcutaneous tissue elsewhere in the rats qu like those described in the genital region of the guinea pig by Dr Cha For this reason the pathogenesis of the so called arthritis may well b the same as that involved in the depot site and in the disseminate granulomata In other words we may be concerned with the metastas of the adjuvant or its components to the subcutaneous tissue of the paws The granuloma formation does not require that an autoimmune mechanism involving articular or other connective tissues be invoked

The life history of the granuloma has not been fully studied by us Initially at the site of injection of the adjuvant there is a nonspecific



Figure 2 Miliary granuloma in inflamed subcutaneous tissue of the same paw as in Figure 1

inflammatory reaction with suppuration about the oil vacuoles. Granulomatous elements were not present on the fifth day after the injection but were in specimens examined on the eighteenth day. The vast majority of the bacilli in the vacuoles had lost their acid fastness 48 hours after injection. In the disseminated tubercles vacuoles were infrequent and bacilli were not identified.

It has been demonstrated experimentally by Hagan and Levine[4] that mycobacteria suspended in paraffin oil injected subcutaneously spread to distant sites—lymph node, spleen, and lungs. Others have observed splanchnic distribution of mineral oil passing through the gastrointestinal tract of rabbits. The route of postulated migration of the adjuvant in the present studies is uncertain—a lymphohematogenous route, at least for the minute components, being likely. Fascial spread in the case of the subcutaneous and paw lesions also is conceivable; there is something simple minded but attractive about the possibility that the digits provide a mechanical barrier allowing local impaction of the oil.

If our thought is correct, we must account for the latent period between the time of injection and the appearance of the "arthritis"—does it reflect the acquisition of altered tissue reactivity or the time required for metastasis? Freund and Lipton observed distant granulomata developing even when the primary depot site was excised as early as an hour after the injection of adjuvant in guinea pigs. Thus at least certain elements of the adjuvant must be discharged from the depot site with great rapidity. Nevertheless, inasmuch as an intense inflammatory reaction appeared at the site of injection into the paw within 24 hours, we assume that the latent period reflects principally a protracted transport time rather than a delayed tissue responsiveness.

The hypothesis that we are dealing with dissemination of the adjuvant mixture would account for Dr. Pearson's evidence that the lesion is not of infectious origin.

The second point worth making has to do with the joint disease per se.

If we return to Figure 1, it is seen that the joint proper is free from inflammation. The joint space contains no exudate and the articular cartilages are intact. There is considerable periosteal new bone, to be sure, enough to cause a false ankylosis, but this is another component of the subcutaneous cellulitis. Capsular and synovial involvement, when present, should also be regarded as secondary to the periarticular cellulitis.

Thus exception may be taken to the term "arthritis" as offered in Dr. Pearson's paper and we have suggested that the experimental lesions arise from the dissemination of the adjuvant or its components. For these reasons, speculations concerning an analogy between these experiments and rheumatoid arthritis appear unwarranted.

Delayed presumably noninfectious inflammation of the paws of rats similar in several respects to Pearson's lesions has been reported following a variety of experimental procedures that are not known to involve hypersensitivity primarily These procedures have included bilateral adrenalectomy in certain strains of rats [1] repeated subcutaneous injections of trypan blue,[3] toxic doses of desoxycorticosterone acetate (DOCA) [6,8] and parabiosis [5] We have repeated some of the experiments (adrenalectomy in several strains of rats injection of trypan blue and of DOCA) without success

It is to be noted too that in hematogenous infectious 'arthritis' of the paws of rats the periarticular tissues rather than the joints proper are affected primarily as in the adjuvant lesions The reason for this is that the primary lesion affects the metaphyseal or subarticular bone marrow and the process finds its way into the soft tissues by eroding the bony cortex This is followed by a spreading cellulitis For this reason any consideration of the infectious etiology of the periarthritis of the paws of small rodents whether due to pleuropneumonia like organisms *Streptobacillus moniliformis* or other bacteria should include minute examination of the bones in these regions — particularly the distal tibia in the hind limb and distal radius at the fore paw

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CHARLES RAGAN (New York New York) Human rheumatoid arthritis lies in an area in which total ignorance of pathogenesis prevails In the

absence of more definitive information we have been forced to resort to the concept that hypersensitivity perhaps plays a role in the development of this disease There is an appreciable volume of evidence in support of this hypothesis resulting from the study of the disease at a clinical level and from its morphologic pathology To summarize this evidence The disease presents in most instances as sustained joint inflammation with acute phase reactants in which no viable agent has been uncovered It is characterized histologically by varying degrees of vasculitis rather nonspecific granuloma formation with resulting overgrowth of connective tissue elements and ultimate scarring in the skeletal structures and not infrequently in viscera as well The one fairly specific lesion is the rheumatoid nodule the genesis of which has been carefully studied by Leon Sokoloff with the conclusion that it originates in vascular tissue It has recently become apparent that maximally severe rheumatoid arthritis may terminate with a picture typical of periarteritis nodosa These two factors have led to the assumption that the element of vasculitis is perhaps preponderant in the pathogenesis of the disease and that the granulomatous changes and scarring result from this primary lesion

To confirm the hypothesis of hypersensitivity an antigen antibody reaction should be demonstrable The sustained nature of the disease implies that an element of autosensitization must be present and the only promising lead now available in human disease concerns the rheumatoid factor Efforts are now in progress * attempting to determine the possible antigenic role of the reaction involving this serologic component which lies in the 19S fraction of gamma globulin and is fairly specific for rheumatoid arthritis systemic lupus erythematosus and periarteritis nodosa It reacts in a variety of agglutination and precipitin reactions with gamma globulin In the precipitin reaction the characteristic feature of the gamma globulin is its state of aggregation A major difficulty in ascribing an antigenic role to this aggregated gamma globulin is its species non specificity since rabbit gamma globulin reacts as well as the human material The development of arthritis similar to rheumatoid arthritis in children with agamma globulinemia and a negative serologic test indicates that the factor is not a universal accompaniment of rheumatoid arthritis Study of the possible role the components of this reaction may play in the pathogenesis of the disease is continuing and at this time no certain answer can be given

If rheumatoid arthritis in man represents hypersensitivity of the delayed type in which the amount of circulating antibody is minimal or non existent the need for a suitable animal model is obvious since man is

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hardly the proper subject for this type of research The rodent arthritis described today by Dr Pearson has many of the characteristics of rheumatoid arthritis from a histologic point of view I find his suggestion attractive that the connective tissue components attracted to the waxy granuloma may be incorporated as antigens into the antibody production at the site I should like to ask Dr Pearson if the rebound phenomenon was ever observed when steroid was withdrawn Also, were repetitive injections used to produce a more sustained type of lesion? There is a great dissimilarity between this rodent arthritis and human disease namely the lack of sustained disease in rodent arthritis This lack has also been apparent in the foreign protein vasculitis so well studied by Germuth The characteristic picture of rodent adjuvant arthritis has been an episode—granted the episode may last 60 days—with resulting deformity whereas in human disease the process may apparently continue for years I am not personally qualified to pass judgment upon the role played by pleuropneumonia like organisms in the genesis of adjuvant arthritis but it does seem likely from Dr Pearson s evidence that these organisms play if any only a minor role in its development If the hypothesis is correct that rheumatoid arthritis represents hypersensitivity involving as the antigen a component of the host we are faced with a frightening task The complexity of the chemical structure of tissue is increasing daily and the mildest isolation procedures may modify immunologic specificity Thus it would appear that the likelihood of detecting an antigen antibody system in the absence of circulating antibody seems remote

GENERAL DISCUSSION

CHAIRMAN CHASE I ask Dr Pearson to close this discussion

DR PEARSON I was most pleased about one element particularly and that was that no one has suggested that this arthritis is due to pleuropneumonia organisms Certainly this is something that has been plaguing me for about two years

As regards the etiology of the arthritis of course this is unsettled up to the moment The *latent period is most interesting* Invariably it is 10½ days and sometimes longer

As I understood it from some of Dr Sokoloff s remarks he feels that direct spread or dissemination of some of the oil and the acid fast bacilli might be factors He also feels as I learned in previous discussion with him that this spread might occur through tissue planes per se out to the peripheral joints

Most of the injections I have given have been into the posterior cervical region It would seem under these circumstances that the material might

reach the front paws before it reaches the hind paws and would set up an initial reaction there The contrary is really true in that the arthritis almost invariably appears in the hind paws much more severely

Furthermore another little point that I did not discuss during the main presentation was that occasionally in the past I have tried injection of a local paw with some of the adjuvant material When I have done this there has been an immediate local response within 24 hours of a rather significant degree with a considerable amount of irritation present at the injection site This almost invariably tends to subside after a few days and then suddenly like the dawn breaking in the morning all four paws on occasion will flare up with an arthritis This does seem to me to be some type of systemic reaction mediated other than by transfer through tissue planes per se

In response to Dr Ragan's question I have tried repeated injections of adjuvant once the arthritis has subsided in a few animals This has been tried in only a very few subjects and it was done about a year and a half ago With this procedure I did not find further recurrence of the arthritis I must say also that in a very few of the animals that I have observed there have been spontaneous recurrences of acute arthritis within 3 to 6 month intervals

Regarding Dr Sokoloff's remark concerning whether one should call this condition an arthritis or not I believe this is a minute and unnecessary point in terminology Certainly we know that in a number of cases of clinical Reiter's disease or rheumatoid arthritis there is really only inflammation in the periarticular tissues and one could if adhering to Dr Sokoloff's criteria strictly label such an entity periarticular rheumatoid disease rather than arthritis

In fact if Dr Sokoloff and his group would look at more than five animals I am sure they would see a number of animals in which there are granulomatous pannus invading the joints and other types of inflammation that cause actual direct adherence of the articular surfaces and not just ankyloses by adjacent periarticular fibrous tissue

CHAIRMAN CHASE I would certainly agree that any criticism that was directed to your mechanism should also be directed to mine and I have reduced the amount of tubercle bacilli for this very purpose without significantly altering results so I feel that the proportionate ratio of organisms in paraffin oil would accentuate the percentage of animals affected if this simple mechanism were to follow

accepted as characterizing allergic reactions that is reactions based on immunologic events They are produced by specific immunization of experimental animals A latent period intervenes between immunization and the appearance of lesions it may be as short as 8 or 9 days and is clearly related to the dose of immunizing antigen After a cycle of lesion formation there may be a remission of the disease process re exposure to antigen in the proper manner at this time results in reappearance of disease after a shortened latent period an anamnestic response (Condie [19]) Immunologic specificity is beautifully illustrated by the elicitation of the inflammatory response only in the tissue corresponding to that used for immunization even central and peripheral nervous system myelin are distinguished though here characteristic immunologic cross reactions may occur [25] The reaction as described in the foregoing paragraphs appears always to be fundamentally the same no matter in which tissue it is incited the perivenous collection of lymphocytes and histiocytes with invasion and destruction of the antigen-containing parenchyma While passive transfer of humoral antibody does not reproduce the lesion in normal recipients, a moderately successful passive transfer of allergic encephalomyelitis with living sensitized lymphoid cells has been achieved [1] Desensitization is not successful but a type of immunologic unresponsiveness may be produced by single injections of antigen in the newborn animal or repeated injections in the adult with loss of the ability to develop disease [2, 8, 7, 20, 25]

The available evidence justifies the identification of the autoallergic diseases as hypersensitive reactions of the delayed or tuberculin type The importance of mycobacteria and the intradermal route of inoculation in the technique of sensitization the nature of the histologic reaction itself the failure of passive transfer with serum and the partial success with living lymphoid cells and finally the susceptibility of the disease process to ACTH and cortisone therapy are all features characteristic of delayed reactions as distinct from cytotoxic anaphylactic or Arthus reactions which are associated with circulating antibody In several autoallergies delayed skin reactions to homologous tissue have been demonstrated which show a partial correlation in time and degree with the disease process and in allergic encephalomyelitis corneal reactions to homologous tissue have been found as well

The Meaning of Autoantigenicity

The terms autosensitization and autoimmunization have been applied not only to the autoallergic diseases under discussion which as we have seen are inflammatory processes probably of the delayed hypersensitive type but also to a group of human diseases, in particular acquired

hemolytic anemia certain chronic leukopenias idiopathic thrombocytopenic purpura paroxysmal cold hemoglobinuria and disseminated lupus erythematosus in which serum factors are found which have serologic properties like those of antibody (reviewed in reference 50) These serum factors appear responsible for some at least, of the manifestations of these diseases While other evidence permitting their identification as autoantibodies is lacking their *in vitro* and *in vivo* behavior can be duplicated experimentally with the use of heteroantisera Autoantibodies are found in additional human diseases, among them viral hepatitis and other liver disease glomerulonephritis and other renal disease myocardial infarction rheumatic fever rheumatoid arthritis other collagen diseases and syphilis, the antigens being found in liver spleen kidney heart muscle joint etc Here there is not the slightest evidence that the antibodies if such they are play a pathogenetic role and except in the case of glomerulonephritis the use of heteroantiserum experimentally fails to produce a lesion resembling that in man Rather it would appear probable that they are formed in response to tissue breakdown in the various disease processes The autoantibodies found in multiple sclerosis chronic thyroiditis sympathetic ophthalmia, and chronic adrenal disease I believe to belong in this category as well

The distinction between the three types of autosensitization considered in the previous paragraph may be accounted for tentatively on the basis of the remoteness of the antigens concerned from the blood stream and consequently from the immune apparatus Tissue antigens which reach the blood stream in negligible quantities if at all whether because of the special nature of their metabolism or because of blood tissue barriers may be regarded by the immune apparatus as foreign substances They can then induce antibody formation or delayed reactivity the latter type of response may result in inflammatory (hypersensitive) reactions to antigen wherever it is present in the tissues that is in autoallergic disease Other tissue antigens less remote from the circulation may be unable to produce delayed sensitivity perhaps because enough antigen is released from time to time to induce a state of partial immunologic nonreactivity like those discussed elsewhere in the present symposium These antigens can however, still elicit the formation of circulating autoantibody when as a result of some destructive process affecting tissue containing the antigen quantities sufficient to immunize are released The antibody in turn can cause disease by its direct effect on antigen containing cells when these are readily accessible as in the case of the blood elements and vascular endothelium More often it fails to produce any lesion at all In the case of glomerulonephritis in which a readily accessible noncellular material is the antigen the lesion produced may be of the Arthus type Finally antigens like serum proteins which are present in the circulation in high

concentration at all times may produce a higher order of immunologic nonreactivity perhaps similar to Felton's immunologic paralysis and are unable to act as autoantigens at all Actually the antigens implicated in the experimental autoallergies are separated from the circulation by striking barriers in many cases the blood brain barrier blood aqueous barrier basement membrane of the seminiferous tubule and acinar epithelium of the thyroid follicle being examples The evidence that autoantigenicity may depend on failure to develop tolerance to an antigen during fetal life because the antigen is absent at the appropriate time [9] is contradicted by the fact that many autoantigens actually are present during fetal life and by evidence that repeated exposure to antigen of adult guinea pigs rabbits or rats depresses these animals ability to develop allergic encephalomyelitis [6, 7, 8] or phaco anaphylactic endophthalmitis [2]

The Relation of the Experimental Autoallergies to Human Disease

The experimental diseases under consideration tend to have a prolonged remittent course since activity of the disease process is determined by the simultaneous presence of antigen normally found in an affected tissue and of sensitivity of the appropriate type above some threshold level The human diseases which resemble the experimental autoallergies (Table I) fall into two distinct groups those in which a single episode with a sharply self limited course occurs and those characterized by a prolonged course with or without clearly distinguishable remissions and exacerbations The latter category includes two cases in which autoimmunization is almost certainly occurring the diseases affecting the lens (phaco anaphylactic endophthalmitis) and the uvea (sympathetic ophthalmia) In both of these groups a traumatic release of tissue antigen provides the immunization which after a suitable interval results in the production of disease this in turn has been shown to be rather well correlated with delayed skin sensitivity to tissue antigen In both a prolonged or remittent course is characteristic A disease picture indistinguishable from that of acute multiple sclerosis may be produced in man by rabies vaccination that is immunization with nervous tissue [3] In chronic polyneuritis chronic thyroiditis cytotoxic contraction of the adrenal and chronic infertility a prolonged or remittent course is seen but without clear evidence for an original immunization with tissue antigen or for the presence of sensitivity Presumably some or all of these must also be due to autosensitization

The group of human diseases which have a self limited course includes encephalomyelitis polyneuritis and uveitis all customarily regarded as

post- or para infectious sequelae of viral infections (chiefly vaccinia measles varicella and rubella) and orchitis normally considered as part of the mumps infection Thyroiditis ovaritis and pancreatitis are less frequent postinfectious sequelae of the same type The self limited character of these diseases their usual association with viral infections especially the exanthems, their failure to recur all are features which suggest the interpretation of these diseases as hypersensitive reactions to virus rather than to tissue antigens It is well to recall that von Pirquet [48, 49] accumulated substantial evidence that the rash in smallpox and measles was probably a hypersensitive reaction to widely disseminated virus More recently it has been demonstrated that delayed sensitivity to mumps virus is present in the infected patient at the time the symptoms of mumps parotitis first appear [46] Is it not reasonable to suppose that the same hyper sensitive response which produces an inflammatory lesion wherever virus is present in for example the skin must also produce one wherever virus is present in the nervous system or other tissues? In favor of this concept is the fact that the interval between viral disease and postinfectious complication is usually too brief for autosensitization to occur The onset of the apparent autoallergy occurs within a few days of the onset of viral disease often simultaneously with it, or indeed in some cases may precede it by a day or two [43] Finley [36] has shown that the onset of 'post vaccinal encephalomyelitis corresponds very closely to the day of maximum reaction at the vaccinated site von Pirquet s acme It should be remarked that phenomena which we have usually been willing to associate with the viral infection as such do not all occur at the same time Fever and the involvement of mucous membranes precede the appearance of the rash by one or more days The rash itself first appears in different areas of the skin on different days Similarly postinfectious neuritis in general starts later than postinfectious encephalomyelitis [43] The relative infre quency of the postinfectious sequelae is not inconsistent with the picture drawn here We do not call generalized vaccinia an autoallergy of the skin because it is uncommon nor has mumps orchitis which occurs in perhaps one out of five cases of mumps been regarded as nonviral for this reason The histological similarity of the lesions in the postinfectious sequelae to those in the autoallergic diseases must be attributed to their being hypersensitive reactions of the same type in the one case to virus and in the other to tissue antigen

Summary

The experimental autoallergic diseases are reproducible experimental entities which appear to constitute a well defined group with constant histologic and other characteristics They provide excellent experimental

models for para infectious diseases in man which are interpreted as hypersensitive reactions to virus and for certain chronic remittent human diseases of unknown origin some of which have been demonstrated to have an autoallergic mechanism

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DESIGNATED DISCUSSION

LEON SOKOLOFF (Bethesda Maryland) Our laboratory * is concerned with experimental joint disease and we have followed Dr Pearson s studies with much interest It is generally recognized that a major stumbling block in approaching the pathogenesis of rheumatoid arthritis and similar disorders is the lack of a suitable experimental model and that research in this area would be greatly facilitated if a reproducible method for inducing a self procreating noninfectious inflammatory joint disease were available

My associate Dr Emanuel Silverstein has repeated some of Dr Pearson s and Dr Stoerk s experiments and has obtained results that confirm their findings in a large part We would however entertain interpretations of the lesions different from those presented in two respects

The experiments are summarized briefly in Table I

TABLE I ARTHRITIS EXPERIMENTS USING FREUND S ADJUVANT DIFCO MYCOBACTERIUM BUTYRICUM

Experiment	Animals			Inoculum				Incidence† Arthritis
	Type	Age	Sex	Type	Dose (ml)	Conc * Myc	Site	
1	Rats (2 3 5)	8–16 mos	M	Spleen	0 2	1	s c neck	0/17
2A	Rats (1 3–7)	8–19 mos	M F	Muscle	0 1	4	s c paw	11/49
2B	Rats (1–7)	8–19 mos	M F	Spleen	0 4	1	s c back	6/35
3A	Rats (8)	2 mos	F	Muscle	0 2 0 4	5	i c paw i c back	0/10
3B	Guinea pigs	5–6 wks	F	Muscle	0 2 0 4	5	i c paw i c back	0/10

Rat strain
1 Albany
2 AxC 9935
3 Buffalo
4 CA/R
5 Fischer
6 M520
7 OM/N
8 Sprague Dawley

s c subcutaneous
i c intracutaneous

Conc myc mg mycobacteria/ml inoculum
† Incidence arthritis number of rats with gross involvement of paw/number of inoculated rats

Sterile mixtures of rat muscle or spleen and Freund s adjuvant with *Mycobacterium butyricum* were employed They were injected by either the subcutaneous or intracutaneous route in the several sites noted in the chart Large amounts of mycobacteria 1 to 5 mg per milliliter were

* National Institute of Arthritis and Metabolic Diseases National Institutes of Public Health U S Public Health Service

present in the inoculum. The experimental animals comprised 8 strains of rats as well as a group of guinea pigs. In the last column it is seen that the frequency of the lesion varied greatly from experiment to experiment but in group A 11 of 49 injected rats developed gross swelling or redness of the paws and in group B 6 of 35. The other experiments were completely unsuccessful.

Histological examination of the affected extremities was carried out in only 5 animals during the acute or active stages of the inflammation; in the remainder it was made after the inflammation had burned out. The microscopic appearance in these paws by and large corresponds to that described by Dr Pearson but in addition 3 of the 5 had another component that we would regard as granulomatous (Figure 1).



FIGURE 1 Subcutaneous inflammatory reaction and periosteal new bone formation in the left tarsal region following injection of adjuvant into right paw. The joint is spared.

This figure is a sagittal section through an inflamed left tarsal region of a rat that had been injected with muscle and adjuvant in the right hind paw. From above down we observe skin, subcutaneous tissue heavily infiltrated with inflammatory cells, then a thick zone of coarse periosteal new bone formation and finally the dorsal portion of the calcaneotarsal joint. Your attention is directed to the cellular infiltrate; there is a diffuse

inflammatory reaction that is best regarded as a cellulitis In addition however there are two pale miliary nodules of a different character

Under higher magnification (Figure) the nuclei of the cells making up the nodules are seen to be elongated and usually somewhat curved The cytoplasm is pale cell outlines are poorly delimited and at times apparently syncytial The cells frequently have a whorled arrangement and appear to be epithelioid cells In other nodules not illustrated here isolated multinucleated giant cells or large round vacuoles were present

We would interpret these lesions as granulomata comparable to those seen at the site of injection of the adjuvant and in the viscera Similar lesions were present in the subcutaneous tissue elsewhere in the rats quite like those described in the genital region of the guinea pig by Dr Chase For this reason the pathogenesis of the so called arthritis may well be the same as that involved in the depot site and in the disseminated granulomata In other words we may be concerned with the metastasis of the adjuvant or its components to the subcutaneous tissue of the paws The granuloma formation does not require that an autoimmune mechanism involving articular or other connective tissues be invoked

The life history of the granuloma has not been fully studied by us Initially at the site of injection of the adjuvant there is a nonspecific

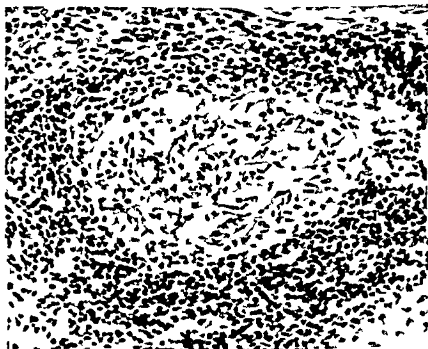


FIGURE 2 Miliary granuloma in inflamed subcutaneous tissue of the same paw as in Figure 1

inflammatory reaction with suppuration about the oil vacuoles. Granulomatous elements were not present on the fifth day after the injection but were in specimens examined on the eighteenth day. The vast majority of the bacilli in the vacuoles had lost their acid fastness 48 hours after injection. In the disseminated tubercles, vacuoles were infrequent and bacilli were not identified.

It has been demonstrated experimentally by Hagin and Levine[4] that mycobacteria suspended in paraffin oil injected subcutaneously spread to distant sites — lymph node, spleen and lungs. Others have observed splanchnic distribution of mineral oil passing through the gastrointestinal tract of rabbits. The route of postulated migration of the adjuvant in the present studies is uncertain — a lymphohematogenous route, at least for the minute components, being likely. Fascial spread in the case of the subcutaneous and paw lesions also is conceivable; there is something simple minded but attractive about the possibility that the digits provide a mechanical barrier allowing local impaction of the oil.

If our thought is correct, we must account for the latent period between the time of injection and the appearance of the "arthritis" — does it reflect the acquisition of altered tissue reactivity or the time required for metastasis? Freund and Lipton[2] observed distant granulomata developing even when the primary depot site was excised as early as an hour after the injection of adjuvant in guinea pigs. Thus at least certain elements of the adjuvant must be discharged from the depot site with great rapidity. Nevertheless, inasmuch as an intense inflammatory reaction appeared at the site of injection into the paw within 24 hours, we assume that the latent period reflects principally a protracted transport time rather than a delayed tissue responsiveness.

The hypothesis that we are dealing with dissemination of the adjuvant mixture would account for Dr. Pearson's evidence that the lesion is not of infectious origin.

The second point worth making has to do with the joint disease per se.

If we return to Figure 1, it is seen that the joint proper is free from inflammation. The joint space contains no exudate and the articular cartilages are intact. There is considerable periosteal new bone, to be sure, enough to cause a false ankylosis, but this is another component of the subcutaneous cellulitis. Capsular and synovial involvement when present should also be regarded as secondary to the periarticular cellulitis.

Thus exception may be taken to the term "arthritis" as offered in Dr. Pearson's paper and we have suggested that the experimental lesions arise from the dissemination of the adjuvant or its components. For these reasons, speculations concerning an analogy between these experiments and rheumatoid arthritis appear unwarranted.

Delayed presumably noninfectious inflammation of the paws of rats similar in several respects to Pearson's lesions, has been reported following a variety of experimental procedures that are not known to involve hypersensitivity primarily. These procedures have included bilateral adrenalectomy in certain strains of rats,[1] repeated subcutaneous injections of trypan blue,[3] toxic doses of desoxycorticosterone acetate (DOCA),[6,8] and parabiosis.[5] We have repeated some of the experiments (adrenalectomy in several strains of rats, injection of trypan blue and of DOCA) without success.

It is to be noted too that in hematogenous infectious arthritis of the paws of rats, the periarticular tissues rather than the joints proper are affected primarily, as in the adjuvant lesions. The reason for this is that the primary lesion affects the metaphyseal or subarticular bone marrow and the process finds its way into the soft tissues by eroding the bony cortex. This is followed by a spreading cellulitis. For this reason any consideration of the infectious etiology of the periarthritis of the paws of small rodents, whether due to pleuropneumonia-like organisms, *Streptobacillus moniliformis* or other bacteria, should include minute examination of the bones in these regions — particularly the distal tibia in the hind limb and distal radius at the fore paw.

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CHARLES RAGAN (New York, New York): Human rheumatoid arthritis lies in an area in which total ignorance of pathogenesis prevails. In the

absence of more definitive information we have been forced to resort to the concept that hypersensitivity perhaps plays a role in the development of this disease There is an appreciable volume of evidence in support of this hypothesis resulting from the study of the disease at a clinical level and from its morphologic pathology To summarize this evidence The disease presents in most instances as sustained joint inflammation with acute phase reactants in which no viable agent has been uncovered It is characterized histologically by varying degrees of vasculitis rather nonspecific granuloma formation with resulting overgrowth of connective tissue elements and ultimate scarring in the skeletal structures and not infrequently in viscera as well The one fairly specific lesion is the rheumatoid nodule the genesis of which has been carefully studied by Leon Sokoloff with the conclusion that it originates in vascular tissue It has recently become apparent that maximally severe rheumatoid arthritis may terminate with a picture typical of periarteritis nodosa These two factors have led to the assumption that the element of vasculitis is perhaps preponderant in the pathogenesis of the disease and that the granulomatous changes and scarring result from this primary lesion

To confirm the hypothesis of hypersensitivity an antigen antibody reaction should be demonstrable The sustained nature of the disease implies that an element of autosensitization must be present and the only promising lead now available in human disease concerns the rheumatoid factor Efforts are now in progress * attempting to determine the possible antigenic role of the reaction involving this serologic component which lies in the 19S fraction of gamma globulin and is fairly specific for rheumatoid arthritis systemic lupus erythematosus and periarteritis nodosa It reacts in a variety of agglutination and precipitin reactions with gamma globulin. In the precipitin reaction the characteristic feature of the gamma globulin is its state of aggregation A major difficulty in ascribing an antigenic role to this aggregated gamma globulin is its species non specificity since rabbit gamma globulin reacts as well as the human material The development of arthritis similar to rheumatoid arthritis in children with agamma globulinemia and a negative serologic test indicates that the factor is not a universal accompaniment of rheumatoid arthritis Study of the possible role the components of this reaction may play in the pathogenesis of the disease is continuing and at this time no certain answer can be given

If rheumatoid arthritis in man represents hypersensitivity of the delayed type in which the amount of circulating antibody is minimal or non existent the need for a suitable animal model is obvious since man is

* At the Department of Medicine Columbia University College of Physicians and Surgeons and the Edward Daniels Faulkner Arthritis Clinic of the Presbyterian Hospital New York Supported in part by US Public Health Service grant

hardly the proper subject for this type of research The rodent arthritis described today by Dr Pearson has many of the characteristics of rheumatoid arthritis from a histologic point of view I find his suggestion attractive that the connective tissue components attracted to the waxy granuloma may be incorporated as antigens into the antibody production at the site *I should like to ask Dr Pearson if the rebound phenomenon* was ever observed when steroid was withdrawn Also, were repetitive injections used to produce a more sustained type of lesion? There is a great dissimilarity between this rodent arthritis and human disease namely the lack of sustained disease in rodent arthritis This lack has also been apparent in the foreign protein vasculitis so well studied by Germuth The characteristic picture of rodent adjuvant arthritis has been an episode—granted the episode may last 60 days—with resulting deformity whereas in human disease the process may apparently continue for years I am not personally qualified to pass judgment upon the role played by pleuropneumonia like organisms in the genesis of adjuvant arthritis but it does seem likely from Dr Pearson s evidence that these organisms play if any only a minor role in its development If the hypothesis is correct that rheumatoid arthritis represents hypersensitivity involving as the antigen a component of the host we are faced with a frightening task The complexity of the chemical structure of tissue is increasing daily and the mildest isolation procedures may modify immunologic specificity Thus it would appear that the likelihood of detecting an antigen antibody system in the absence of circulating antibody seems remote

GENERAL DISCUSSION

Chairman Chase I ask Dr Pearson to close this discussion

Dr Pearson I was most pleased about one element particularly and that was that no one has suggested that this arthritis is due to pleuropneumonia organisms Certainly this is something that has been plaguing me for about two years

As regards the etiology of the arthritis of course this is unsettled up to the *moment The latent period is most interesting* Invariably it is 10½ days and sometimes longer

As I understood it from some of Dr Sokoloff s remarks he feels that direct spread or dissemination of some of the oil and the acid fast bacilli might be factors He also feels as I learned in previous discussion with him that this spread might occur through tissue planes per se out to the peripheral joints

Most of the injections I have given have been into the posterior cervical region It would seem under these circumstances that the material might

reach the front paws before it reaches the hind paws and would set up an initial reaction there The contrary is really true in that the arthritis almost invariably appears in the hind paws much more severely

Furthermore another little point that I did not discuss during the main presentation was that occasionally in the past I have tried injection of a local paw with some of the adjuvant material When I have done this there has been an immediate local response within 4 hours of a rather significant degree with a considerable amount of irritation present at the injection site This almost invariably tends to subside after a few days and then suddenly like the dawn breaking in the morning all four paws on occasion will flare up with an arthritis This does seem to me to be some type of systemic reaction mediated other than by transfer through tissue planes per se

In response to Dr Ragan s question I have tried repeated injections of adjuvant once the arthritis has subsided in a few animals This has been tried in only a very few subjects and it was done about a year and a half ago With this procedure I did not find further recurrence of the arthritis I must say also that in a very few of the animals that I have observed there have been spontaneous recurrences of acute arthritis within 3 to 6 month intervals

Regarding Dr Sokoloff s remark concerning whether one should call this condition an arthritis or not I believe this is a minute and unnecessary point in terminology Certainly we know that in a number of cases of clinical Reiter s disease or rheumatoid arthritis there is really only inflammation in the p rarticular tissues and one could if adhering to Dr Sokoloff s criteria strictly label such an entity periarticular rheumatoid disease rather than arthritis

In fact if Dr Sokoloff and his group would look at more than five animals I am sure they would see a number of animals in which there are granulomatous pannus invading the joints and other types of inflammation that cause actual direct adherence of the articular surfaces, and not just ankyloses by adjacent periarticular fibrous tissue

CHAIRMAN CHASE I would certainly agree that any criticism that was directed to your mechanism should also be directed to mine and I have reduced the amount of tubercle bacilli for this very purpose without significantly altering results so I feel that the proportionate ratio of organisms in paraffin oil would accentuate the percentage of animals affected if this simple mechanism were to follow



XII

Some Factors Modifying the Response to Allergens

Chairman MERRILL W. CHASE, PH.D. (*New York, New York*)

45

The Hereditary Predisposition in Man to Develop Hypersensitivity A Critical Review

PAUL F deGARA MD

(New York New York)

The familial occurrence possibly hereditary in nature of manifestations of hypersensitivity has been known for a long time One of the first observations dealing with asthma in several members of the family of a physician was published over three hundred years ago by Sennertus[54] Thereafter between 1650 and 1900 a number of reports on the familial or hereditary occurrence of allergic conditions appeared in the British French German and American medical literature

During the past fifty years the subject has been extensively studied Observations have been reported on pedigrees of families with allergic manifestations in several successive generations and on hypersensitivities in twins In addition, statistical data and experimental work in pathology and immunology as well as genetic analyses have been published In this presentation no attempt will be made to review the whole literature inasmuch as certain phases are well summarized in various treatises or monographs[8, 16, 2, 40, 44, 53, 5, 9] Instead a number of representative publications will be critically examined

Allergic reactions are either of the immediate (urticarial) or of the delayed (tuberculin) type but the two types are not always sharply separable[19] At the Eleventh Annual Meeting of the American Academy of Allergy in 1956 Chase discussed the mechanism of sensitization He pointed out that in studies with chemical allergens in guinea pigs the two types of allergic reactions may appear at the same time though one may predominate over the other Therefore no attempt will be made to separate in this presentation the hereditary predisposition to develop hypersensitivity reactions to one or the other type (See also references 30 and 31)

Pedigrees of families either with identical or with a variety of symptoms of hypersensitivity among many members of several generations have been reported[13, 15, 26, 25, 8, 33, 35, 37, 59, 62] Two typical graphs are presented with the kind permission of authors and publishers The first deals

with a family observed by Crowder and Crowder [28] in a small Midwestern community Angioneurotic edema was noted among 27 of 51 descendants in four generations (53 per cent) In the other group reported by Crawford of England [6] 18 of 27 members (66 6 per cent) of five generations had various forms of allergies In giving me permission to use this chart Dr Barbara Crawford of Leamington Spa England has informed me in a letter dated January 25, 1958 that since publishing it more than twenty years ago the young son of in the fifth generation has proved to be subject to urticaria Thus in this family allergic manifestations were observed in six successive generations

It must be remembered that pedigrees are acceptable only if all the affected members were personally examined by the observer However very few reports in the literature fulfill this essential condition

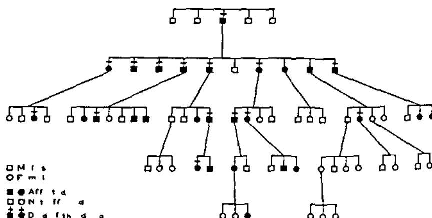


FIGURE 1 Familial angioneurotic edema From Crowder J R and Crowder T R Five generations of angioneurotic edema *A M.A Arch Int Med* o 840 1917

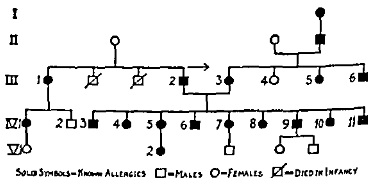


FIGURE 2 Allergy in five generations of a family group From Crawford B G R Allergy in five generations *Brit M J*, 1 751 1936

While investigations of twins play an important role in genetic studies only a few reports have been published[7, 14, 2, 2, 55, 58, 60, 69] in relation to hypersensitivity and of these, only two deal with sizable groups[7, 60] In 1936 two German physicians[60] personally examined 71 pairs of twins Their findings on 59 pairs having various allergic manifestations are summarized in Table I

TABLE I Occurrence of Allergy in Twins*

Type of Twins	Number of Pairs	Concordance of Allergy Number	Concordance of Allergy Per Cent	Concordance of Symptoms Number	Concordance of Symptoms Per Cent
Identical	34	30	88 ?	20	58 8
Fraternal	25	16	64 0	5	20 0

According to Spaich and Ostertag

These results would indicate that a high degree of concordance of allergy and even of symptoms (meaning the shock organ) exists in the group of identical twins On the other hand a recent American[7] report on 59 pairs of identical twins failed to substantiate the results of the German investigators

In view of the importance of twin studies in heredity and of the small number of available data it would appear that additional investigations are needed before conclusions can be drawn

Since the first large series was presented in 1916[25] numerous statistics have appeared in the American and European medical literature on the importance of hereditary factors in diseases due to hypersensitivities The significance of the family history and of the age of onset of allergic manifestations has been emphasized[3, 5, 10, 5, 61] Thus a positive family history could be obtained in over one half of the patients with allergic conditions and only in less than one tenth of nonallergic controls[65] Furthermore with a history of hypersensitivities on both the paternal and maternal sides a greater number of offspring became affected and the first symptoms appeared at an earlier age It was found that no definite or specific sensitization is inherited merely the capacity of becoming hypersensitive is transmitted[3, 25, 57] Other reports pointed out that atopic shock tissues may be subject to a separate hereditary influence[18, 20] The crucial role of environmental factors in evoking symptoms of hypersensitivity has also long been recognized[8, 19, 20] However while Coca[20] believes that in the absence of the hereditary factor, contact with an atopen does not produce atopy others suggested[58, 62] that allergy may be either on a hereditary basis or acquired This viewpoint was re emphasized in a recent publication[51]

These reports concerning the importance of a positive family history and of the age factor were repeatedly confirmed both here and abroad [3, 5, 9, 11, 1, 3, 4, 3, 39, 50, 5, 53, 61] It must be remembered however that the agreement is far from being unanimous and that several dissenting reports have been published [1, 1, 43, 48, 66] It is also interesting to find the following statement in one of the most recent textbooks [57] on allergy The scarcity of impressive new studies of the problem in recent years may no doubt be attributed to the fact that the importance of heredity is so generally accepted that further compilation of statistics is considered fruitless by most investigators '

A critical scrutiny of the published reports and of personal observations [9] leads to the following remarks Many reports lack adequate control studies Many if not most of the publications are based on histories obtained from patients obviously intelligence and reliability differ widely among the groups used by various investigators It is noteworthy that only one report specifically states that histories were obtained only after the patients or their parents had become thoroughly aware of and familiar with the allergic problems (preferably after one to two years of exposure to the allergist [39]) Furthermore, there is considerable difference concerning the criteria used in various places as to which manifestations should be included in the respective reports on hypersensitiveness

Evidence so far reviewed leaves little doubt that hypersensitivity in man is familial in nature However the exact mechanism of its transmission has been and still is a matter of scientific controversy [34] Coca and Cooke [1] in 19 3 and numerous writers thereafter have maintained that atopy is inherited on a genetic basis On the other hand some authors especially Ratner [4, 45] took a different viewpoint and asserted that protein hypersensitiveness in man is transmitted from mother to offspring by way of the placenta Twenty years later in 195 this author still rejected the possibility of heredity as a factor in predisposing man to develop hypersensitivity [46] However only one year later he concluded that the available data do not prove (nor exclude) that the capacity to become sensitized is controlled by genes [4] While it is a known fact that the human placenta is permeable for a number of antibodies this could not be demonstrated by various investigators [6, 66, 70] for skin sensitizing antibodies of atopic conditions The idea of placental transference was also refuted by published reports on allergy in identical twins [7]

In their first publication on human sensitization Cooke and Vander veer [5] concluded that predisposition to hypersensitiveness is inherited according to the Mendelian laws and governed by a dominant gene Mendelian dominance was claimed to be the mode of inheritance by others [3, 9, 13, 21, 32, 49, 59, 62] but methods and materials used in most instances were inadequate A few investigators suggested that a recessive gene may

be responsible.[63, 64] The critical reviewer must admit that absolute conformity with the Mendelian laws has not yet been proved. At present the article by Wiener and associates[71] published in 1936 still seems to offer the best explanation for this complicated problem. According to this theory two allelomorphic genes are responsible. One designated H is the nonallergic and the other called h is allergic. The three genotypes resulting from their combinations are HH where there is neither hypersensitivity nor transmission of a predisposition to develop allergy, hh which represents the true allergic who develops symptoms of hypersensitivity before reaching puberty and finally Hh or hH in which allergies may develop after puberty or no symptoms will appear but hypersensitivities may be transmitted to descendants. On the basis of this

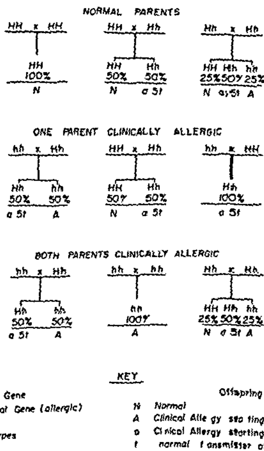


FIGURE 3. Inheritance of allergy. (After Wiener, Zieve and Fries.)

theory a chart showing the various possibilities is presented The upper part gives the results of matings between clinically nonallergic persons Where both parents are pure HH all offspring will likewise be pure HH namely without manifestations of hypersensitivity throughout life and without transmission of allergy One normal HH parent married to another normal (but Hh) person will produce 50 per cent HH and 50 per cent Hh children, of these one sixth may develop allergies later in life and the remaining five sixths may be transmitters only of allergic tendencies to their respective offspring The last group composed of two Hh parents will have only 5 per cent HH children 25 per cent will be definitely allergic and the remaining 50 per cent will be Hh and follow the pattern described for the preceding group The middle columns deal with various combinations resulting from marriages between one definitely allergic parent to a normal partner The resulting descendants will belong to one of the groups described depending on the resulting genotypes *Finally if both parents have symptoms of hypersensitivity as shown* in the lower part of the chart the number of allergic members among the offspring will be larger than in any other group and that of normals will be the smallest

This theory gives a satisfactory explanation for the fact that allergic persons may have normal ancestors and normal offspring may be the result of the mating of two persons with definite symptoms of hypersensitivity (see also reference 6)

The inheritance of hypersensitivity tendencies in four generations of a typical American family [8] is shown in Figure 4

In the experimental field important differences between atopic and nonatopic persons in the response to injections of diphtheria toxoid

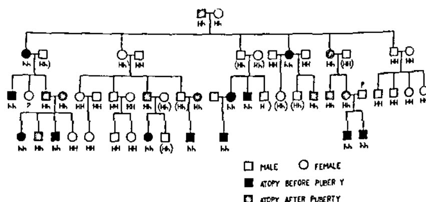


FIGURE 4 Occurrence of atopic allergy in an American family with hypothetical genotypes showing how the inheritance might be accounted for on the basis of Wiener's theory From Boyd W C *Fundamentals of Immunology* Copyright 1956 by Interscience Publishers Inc., New York

have been reported[30] Individuals with hypersensitiveness produce antitoxin with good neutralizing properties but without precipitating ability as compared with antitoxin manufactured by nonallergic persons This non-precipitating antibody possesses most of the properties of the atopic reagin This line of approach may open new avenues for studies on the subject

The difficulties encountered in this field are well illustrated by an interesting study on the genetic control of the response to antigenic stimuli in crossbred rabbits[31] It was found that the level of the secondary antibody response to the relatively simple tobacco mosaic virus is a highly heritable characteristic On the other hand no such 'heritability' could be detected when a more complex antigen such as bovine plasma albumin was used Apparently the complexity of the antigen makes an estimation of heritability very difficult if not impossible

Conclusions and Recommendations

A critical survey of the literature on the hereditary aspects of hypersensitivity leads to the following conclusions

Allergic conditions are familial diseases Studies on twins and pedigrees of families and statistical and experimental results seem to indicate that genetic factors play an important role in predisposing an individual to react with certain manifestations following exposure to allergenic substances However the observations in the majority of the reports are not satisfactory for one reason or another Of the various genetic hypotheses the theory by Wiener and his associates published over twenty years ago still appears to offer the most convincing answer for certain otherwise inexplicable facts

On the basis of these conclusions, the following recommendations seem to be warranted

A well planned thorough investigation on the subject is needed Such a study including a large number of controls should be done on numerous family groups and should be started by a group of comparatively young investigators A physician who at 30 years of age has the opportunity of personally examining all the members of at least three generations of a family may, during the ensuing thirty years come into personal contact with at least two additional generations of descendants Thus he would be able to report firsthand information on five to seven generations of the same family group There is no doubt that such a study with the focus of attention directed toward the descendants rather than the ancestors could lead to a satisfactory solution of the problem

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Some Factors Affecting Contact Sensitization in Man

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Were it not for the guinea pig little of fundamental importance would be known about allergic contact dermatitis The biological principles governing this allergic state have been almost uniquely a product of guinea pig research A vast clinical experience with contact sensitization in man has served merely to confirm and amplify this knowledge The sensitization processes are the same However the human offers certain experimental advantages Two features are noteworthy (1) The human has a greater susceptibility to contact sensitization and (2) the inflammatory expression is sharper and more distinctive

As regards the first there is ample circumstantial evidence that humans become sensitized to innumerable agents that lack the capacity to sensitize guinea pigs Only the most powerful sensitizers can be used in guinea pig work Even with these the effects are less consistent than with humans Far more important than the limited number of contact sensitizers is the relative weakness of the allergic state in guinea pigs This ordinarily cannot be overcome simply by intensifying the stimulus Strong concentrations often bordering upon irritativeness must be used to elicit the dermatitis For all but the most experienced and careful workers this is a source of uncertainty and downright error Chase has rightly emphasized the necessity for great care in interpreting responses of such delicacy [5] By extraordinary means he succeeded in sensitizing guinea pigs to picryl chloride more strongly than ever before so that patch tests with 1 15 000 and sometimes 1 45 000 elicited reactions This record result is to be contrasted with the common ability of human subjects to react to 1 1 000 000 concentrations of dinitrochlorobenzene after being sensitized by a single application Bloch a pioneer in experimental human and guinea pig sensitization became so allergic to Primula that he could not walk into the room where the plants were being ground without developing a dermatitis [2] Truly exquisite degrees of poison ivy sensitivity are common [11] Some nurses are so contact sensitive to penicillin that they cannot be in

attendance on patients receiving injections or handle the unopened vials

The second advantage of the human is the much sharper dermatitis Vesiculation is the distinctive hallmark This is typically absent in the guinea pig Maximal human reactions show large blisters oozing crusting swelling and bright erythema These responses scale downwards through all degrees to the minimal expression of redness and slight edema Patch tests of course are applied to the glabrous skin Hairy regions such as the scalp are less reactive corresponding to the furred animal In the latter redness and edema ordinarily constitute the total expression In minor degrees these are difficult to assess The upshot of all this is that one has a narrow zone to work with in guinea pigs Reactions fall off rapidly with dilution In humans on the other hand the dermatitis can be elicited over a wide range of concentrations as a result of stronger sensitization Furthermore the very vividness of the dermatitis and the play of pathologic changes provide a broad range which makes grading far sharper and more certain

With proper regard for the vagaries of patch testing one can construct for humans sensitization curves by plotting the frequency of sensitization against concentration [7] With norms thus established, agencies which influence the sensitization rate can be assessed experimentally Typical curves are shown in Figure 1 for 2 4 dinitrochlorobenzene (DNCB) and

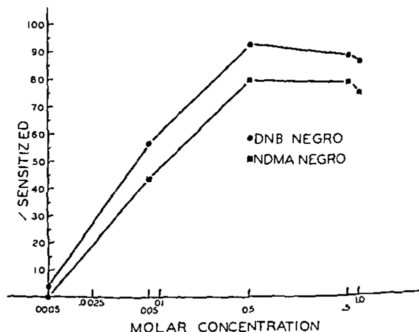


FIGURE 1 Frequency of sensitization to varying concentrations of dinitrochlorobenzene and p nitrosodimethylaniline

p nitrosodimethylaniline (NDMA). Sensitization is accomplished by dissolving the agents in acetone and slowly delivering 0.5 ml on the forearm skin within an open cup 2.9 cm in diameter. A stream of air hastens evaporation of the acetone. The site is covered with a bandage. This technique eliminates the variable effect of oily vehicles. Only a single application is required. Within limits the rate of sensitization is not increased by a larger number of applications made at the same time, that is, simply by increasing the area of exposure. There is a threshold concentration. Below this no amount even if applied to the total normal skin surface will sensitize.

For the past several years we have been investigating contact dermatitis in incarcerated populations of human volunteers, mainly prisoners. The list of forces which can alter the capacity to become sensitized is large. Here we shall confine our attention to two phenomena, one of which increases and the other decreases sensitizability to contact allergens.

The Interference Phenomenon

An individual may be simultaneously sensitized to a number of unrelated agents. In our studies we commonly applied two to four substances at the same time. The individual rates of sensitization apparently remained the same whether these were applied alone or together. However, there was one exceptional situation in which a strong allergen blocked sensitization to a weaker one.[7] The circumstances were quite special. In Figure 1 one can observe that DNCB is a stronger sensitizer than NDMA in that similar concentrations sensitize a larger number. If DNCB, the stronger allergen, is applied in a concentration one hundred times that of NDMA, sensitization to the latter is considerably reduced, whereas that of DNCB is in no way affected (Table I). Attention is called to the fact that the

TABLE I. Interference of p Nitrosodimethylaniline by Dinitrochlorobenzene

Allergen	Expected Incidence When Applied Alone	Actual Incidence When Applied Simultaneously
DNCB (0.5 M)	87% (107/120)	78% (18/23)
NDMA (0.005 M)	45% (62/137)	14% (3/23)

Note that the concentration of DNCB is one hundred times that of NDMA and that its sensitization frequency is not changed by being applied simultaneously with NDMA. By contrast, sensitization to NDMA is markedly reduced.

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DNCB is still in evidence when the for
possibly even 30 days later?

Now suppose the tables are turned so th
is one hundred times that of DNCB No i

TABLE II ABSENCE OF INTERFERENC

Allergen	Expected Incidence When Applied Alone
DNCB (0 005 M)	62% (65/105)
NDMA (0 5 M)	79% (42/53)

There is no blocking of DNCB sensi
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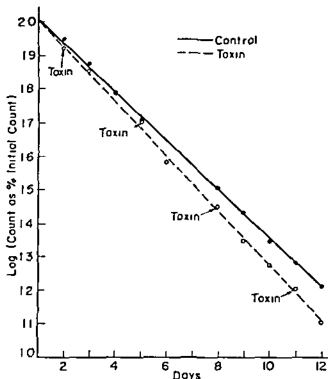


FIGURE 5 Effect of repeated injections of diphtheria toxin on the release of I^{131} from the thyroid glands of guinea pigs

injection of bacterial exotoxins depresses and those in which it increases thyroid activity this division was based solely on the effect of various bacterial exotoxins No attempt was made to analyze the mechanism of stimulation In Figure 7 are shown the effects on the activity of the thyroid gland 24 hours after the intradermal injection of diphtheria toxin or toxoid Toxin was injected into normal guinea pigs toxoid into animals previously sensitized with alum precipitated diphtheria toxoid Thus the comparison is one of toxicity versus hypersensitivity to see whether comparable sized areas of damage in the skin produced comparable effects on the activity of the thyroid gland

The area of damage is shown in terms of the mean of lesion diameter measured 4 hours after the injections The activity of the thyroid gland 24 hours after the injections is expressed in terms of percentage fall in radioactivity from the initial value As the dose of diphtheria toxin is increased to the fatal dose there is a corresponding though feeble increase in thyroid activity In the comparable group of sensitized guinea pigs the slope of the dose response is steeper as the dose of toxoid is increased

agents are applied at different sites The blocking effect does not require that the allergens be applied at the same time Interference of NDMA by DNCB is still in evidence when the former is applied 5 10 15 and possibly even 30 days later[7]

Now suppose the tables are turned so that the concentration of NDMA is one hundred times that of DNCB No interference results (Table II)

TABLE II Absence of Interference of DNCB by NDMA

Allergen	Expected Incidence When Applied Alone	Actual Incidence When Applied Together
DNCB (0 005 M)	62% (65/105)	60% (9/15)
NDMA (0 5 M)	79% (42/53)	73% (11/15)

There is no blocking of DNCB sensitization even though it is applied in a concentration one hundred times weaker than NDMA

The weaker sensitizer cannot be made to block the stronger one by manipulating the concentrations

We have been able to show that 0 5 molar DNCB reduces the sensitization rate to monobenzyl ether of hydroquinone from an expected incidence of 23 per cent to 8 per cent The blocking effect to DNCB is manifested only when it is used in sufficient strength to give maximum frequencies of sensitization that is when the antigenic stimulus is intense

The question arises whether substances other than DNCB can exert blocking action Again it is a matter of finding a suitable combination of agents of intrinsically unequal sensitizing capacity NDMA and formaldehyde are such a pair For sensitization a small paper disc soaked in 2 per cent Formalin solution was applied to a site freshly irritated by freezing and occluded under an impermeable dressing Applied alone the incidence of Formalin sensitization is 29 per cent This figure falls to 6 per cent (2/31) when applied simultaneously with NDMA Preliminary experiments indicate that NDMA can block sensitization to fluidextract of krameria The principle seems a general one

Suppose that one selects a pair of sensitizers neither of which is a powerful allergen but one of which is still considerably stronger than the other? Monobenzyl ether of hydroquinone and 1 hydrazinopthalazine (Apresoline) fulfill this requirement The individual rates of sensitization are 30 per cent and 9 per cent respectively The method consisted of applying a 10 per cent concentration of each agent in Carbowax ointment for a 48 hour period This was repeated at a new site two more times allowing a 2 day rest period between applications When both were

TABLE III Enhancement of Sensitization to Monobenzyl Ether of Hydroquinone by Various Kinds of Irritation

Control	Freeze	Cantharidin	Ultraviolet Light	Burns
5% (2/40)	35% (6/17)	20% (14/66)	16% (3/19)	18% (3 17)

if the reactions are of comparable degree and evolve at about the same time rate Freezing is easiest cleanest most acceptable to the subject and gives fairly reproducible results We have employed it in preference to other techniques Attention is called to the fact that in the study I have just described the skin is grossly normal at the time the sensitizer is applied The inflammatory reaction develops in the presence of the sensitizer

A somewhat different way of demonstrating the augmenting effect of irritation is to apply nonsensitizing nonirritating concentrations of NDMA to sites undergoing an inflammatory reaction A single application of 0 5 ml of 0 0005 molar NDMA in acetone did not sensitize any of 4 control subjects however when 0 [illegible] per cent cantharidin was incorporated in the same solution to produce irritation the same application sensitized 7 of 15 volunteers (46 per cent) This is about the rate expected of a ten times stronger NDMA solution applied to normal skin

The manner in which irritation promotes sensitization is unknown It is not merely a matter of enhanced absorption If that were so intradermal *injection should be a highly effective technique* However with certain sensitizers such as monobenzyl ether of hydroquinone intradermal injection is far less satisfactory than topical application In humans experience teaches that application to the surface is as a rule a more efficient *method of sensitizing than by injection especially with the less powerful* sensitizers Irritation it should be noticed will not confer sensitizing ability on agents which lack this power Furthermore while most powerful sensitizers are irritants this is not an absolute prerequisite p phenylenediamine for instance is a potent nonirritating allergen Obviously most irritants are not sensitizers Sensitization and irritation are independent variables However if a sensitizer happens to be an irritant this improves its sensitizing capacity in the same way that any irritation howsoever produced is favorable for sensitization

Time Relationships

In the studies I am reporting the sensitizer had the opportunity to act *during the time* the skin passed from a normal to an inflamed state Two issues arise (1) what is the effect of irritating the skin at varying times after the sensitizer has been applied and conversely ([illegible]) what happens when the sensitizer is applied to skin which is already in an inflamed state

As regards the former, Haxthausen's results[9] are surprising to say the least. By freezing the skin with dry ice for 10 seconds at varying times after applying dinitrochlorobenzene he completely prevented sensitization. Freezing exerted this suppressive action even after 8 days! It should be added that a 10 second application of dry ice does not produce necrosis or even strong inflammation. This question was studied in the following way: 0.5 ml of 0.5 molar DNCB, which sensitizes about 90 per cent of adult Negro males, was applied by the cup method. This concentration is in itself irritating. The inflammatory reaction was then greatly aggravated by a 60 second freezing with Frigiderm spray at the following intervals after application of the sensitizer: 30 minutes (8 subjects), 24 hours (5 subjects), and 48 hours (4 subjects). Every subject became sensitized. If anything, a subsequent strong inflammatory stimulus aids, not antagonizes, sensitization. Haxthausen's findings are utterly incongruous with the fact that total excision of the application site a day or more later does not prevent sensitization in guinea pigs.[8] In humans we found that complete excision down to the subcutaneous fat after 32 hours does not block sensitization to 0.5 molar DNCB; indeed, excision within 6 hours after application does not prevent sensitization in some subjects. Brief residence of the sensitizer within the skin seems adequate.

More interesting relationships are revealed by the contrasting experiment in which the sensitizer is applied to already inflamed skin. This is the situation which clinical experience has judged to be highly predisposing to sensitization. First let us consider severe inflammation produced by a 60 second Freon 12 freezing. Concentrations of DNCB and NDMA sufficient to sensitize about 50 per cent of subjects were applied to sites frozen 2, 10 and 30 days earlier. By 10 days the sites were clinically healed but still showed inflammatory sequelae such as pigmentation and scaling. Table IV indicates that there was possibly some decrease in the sensitization rate after 2 days, marked suppression after 10 days, and still significant interference after 30 days. That this result is not due to lack of

TABLE IV. EFFECT OF APPLYING CONTACT SENSITIZERS AT VARIOUS TIMES AFTER A PRIOR SEVERE IRRITATION PRODUCED BY FREEZING FOR 60 SECONDS

Allergen	Control	Day Allergen Applied After Freezing for 30 Seconds with Freon 12		
		2	10	30
DNCB (0.005 M)	61% (70/114)	44% (4/9)	12% (1/8)	
NDMA (0.005 M)	45% (62/137)	33% (3/9)	11% (3/27)	25% (3/12)

There is a decrease in sensitizability after [illegible] 10 and 30 days.

absorption was demonstrated by injection of the sensitizer the same depression was evident It would appear from this that the clinical dictum requires some amending or qualifying Skin which has recently been the site of acute inflammation or has recently recovered therefrom is less sensitizable One cannot conclude from such acute studies whether sites of chronic dermatitis in disease states are more resistant to sensitization but our preliminary studies suggest that this is the case These findings conflict with those of Sulzberger *et al* who found a high rate of sensitization to sulfonamide creams which were applied to third degree burns after debridement was complete Our experience would indicate that such severely traumatized skin would be quite resistant to sensitization Perhaps sensitization developed from contact with the surrounding normal skin since no effort was made to confine the medicament strictly to the burned sites It should be noted that only a third of those clinically judged to be sensitive were actually patch test positive

Table V indicates that even a mild irritation suppresses sensitizability

TABLE V Effect of Prior Mild Irritation (3 Second Freeze) on Contact Sensitization

Allergen	Control	Day Allergen Applied After Freezing for 3 Seconds 2	10
DNCB (.005 M)	61% (70/114)	78% (7/9)	45% (5/11)
NDMA (.005 M)	46% (63/137)	58% (7/12)	18% (6/32)

Even this gentle trauma diminishes sensitizability at 10 days

to DNCB and NDMA applied 10 days later when the skin is almost normal grossly

What actually happens in the crucial few hours after the sensitizer contacts the skin is unknown Accordingly there is no explanation of how irritation either promotes or depresses sensitization depending on the time the sensitizer is applied with relation to the age of the irritation

Summary

1 Competition of contact sensitizers has been demonstrated in humans A potent allergen such as dinitrochlorobenzene blocks sensitization to weaker sensitizers applied simultaneously or within weeks of the time of application of the blocking allergen Weaker sensitizers cannot interfere with sensitization to stronger ones

. Irritation promotes sensitization when the allergen is applied to a freshly inflamed site The opposite effect occurs when the allergen is applied to an area irritated 10 days earlier Skin which has recovered from a prior inflammation is less sensitizable

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*The Metabolism of Different Species in Relation to Their Response to Allergens**

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The species of animal commonly studied by the research worker can be divided into two groups by the response of their thyroid glands and adrenal cortices to the injection of bacterial exotoxins.[2] In one group the activity of the thyroid gland is depressed and there is a reciprocal relationship between the activity of this gland and that of the adrenal cortex. In the other group stimulation of both glands occurs simultaneously. Since in general adrenocortical stimulation decreases and thyroid stimulation increases sensitivity to allergens,[7, 8] these changes in the relative activity of the two glands will influence the intensity of damage due to bacterial allergens.

Harris and his colleagues proved that stress depressed thyroid activity in the rabbit and rat. In addition they showed that in the adrenalectomized rabbit thyroid activity is depressed as the concentration of adrenal steroids in the blood is increased. Likewise my colleagues and I found that bacterial exotoxins depressed thyroid activity in rabbits (Figure 1), in rats (Figure) and also in mice (Figure 3). In these experiments the I[131] output method was used: the rate of release of thyroidal radioactivity follows an exponential curve, and decreased or increased activity of the gland is associated with a decreased or increased slope of the curve respectively. In keeping with the reciprocal hypothesis of Harris, depression of thyroid function following the injection of exotoxin was reversed by injection of thyrotrophin.

In direct contrast, the injection of an exotoxin stimulates thyroid activity in the rhesus monkey (Figure 4) and in the guinea pig. The thyroid gland of the guinea pig responds feebly to change in temperature and even to the injection of thyrotrophin. Repeated injections of small doses of toxin were needed to produce slight though statistically significant stimulation of thyroid activity (Figure 5). For the moment I am con-

* The work reported in this article was in part supported by U.S. Public Health Service Research Grant E 169.

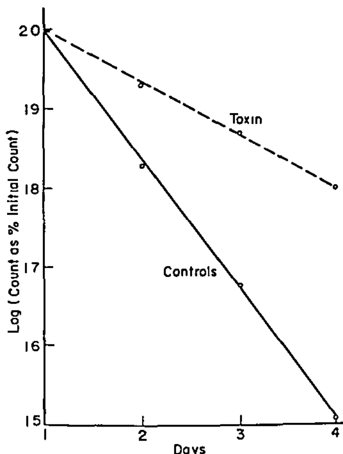


FIGURE 1 Effect of diphtheria toxin (LD$_{0}$) on the release of I^{1} from the thyroid glands of propylthiouracil treated rabbits

cerned less with the size of the effect than with proving that the reciprocal relationship of Harris does not apply to the guinea pig or rhesus monkey I believe that it does not apply to man Harris and Woods in an attempt to explain the long standing clinical association between stress and hyper thyroidism suggest that the normal human as well as laboratory animals responds to emotional or physical stress with increased activity of the adrenal cortex They postulate that if the adrenocortical response fails to occur and the inhibiting effect of a raised blood concentration of adrenal steroids of thyrotrophin is lacking thyroid hyperactivity may ensue

There is a possible alternative explanation The rhesus monkey and guinea pig are resistant to many of the actions of cortisone[6,8,11] they maintain body weight (Figure 6) gamma globulin synthesis and anti toxin production under cortisone administration their resistance to bac

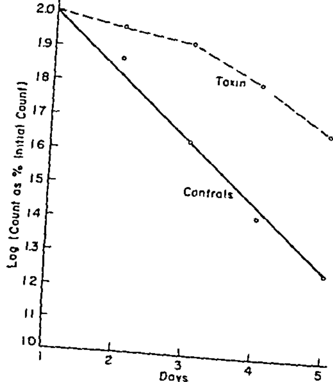


FIGURE 2 Effect of Shiga toxin (LD$_{50}$) on the release of I^{131} from the thyroid glands of propylthiouracil treated rats

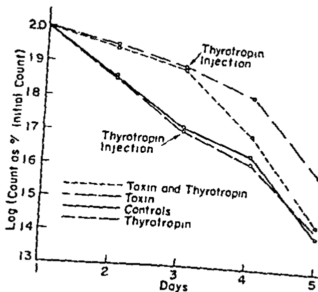


FIGURE 3 Effect of Clostridium septicum toxin on the release of I^{131} from the thyroid glands of propylthiouracil treated mice

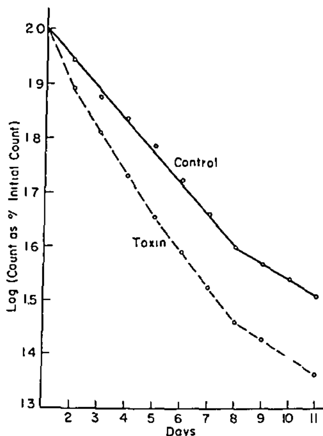


FIGURE 4 Effect of Clostridium septicum toxin on the release of I^{131} from the thyroid glands of rhesus monkeys

terial infection is not significantly depressed by cortisone even when it is injected in large doses On a protein containing diet their nitrogen balance can be maintained even though cortisone is injected daily in a dose of 50 mg per kilogram In contrast the rat rabbit and mouse are sensitive to all these effects of cortisone It may be that in what I have called cortisone resistant species [10] thyroid function is not depressed as the level of blood steroids rises because such species are resistant to this action of cortisone rather than because there is some hypothetical failure of adrenal function

Because of the close analogy between the guinea pig and man (Table I) [6 8] further studies have been carried out on this animal both at the National Institute for Medical Research with my colleagues Dr R Pitt Rivers and Mrs J Gerwing and also at the University of Pittsburgh with Professor Hubert Bloch

Although the species mentioned can be divided into those in which the

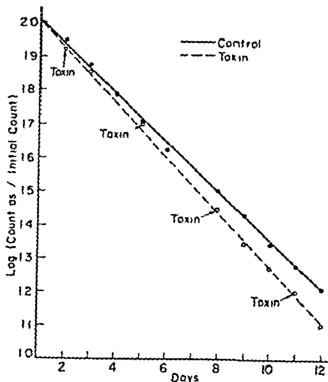


FIGURE 5 Effect of repeated injections of diphtheria toxin on the release of I^{131} from the thyroid glands of guinea pigs

injection of bacterial exotoxins depresses and those in which it increases thyroid activity this division was based solely on the effect of various bacterial exotoxins No attempt was made to analyze the mechanism of stimulation In Figure 7 are shown the effects on the activity of the thyroid gland 4 hours after the intradermal injection of diphtheria toxin or toxoid Toxin was injected into normal guinea pigs toxoid into animals previously sensitized with alum precipitated diphtheria toxoid Thus the comparison is one of toxicity versus hypersensitivity to see whether comparable sized areas of damage in the skin produced comparable effects on the activity of the thyroid gland

The area of damage is shown in terms of the mean of lesion diameter measured 24 hours after the injections The activity of the thyroid gland 4 hours after the injections is expressed in terms of percentage fall in radioactivity from the initial value As the dose of diphtheria toxin is increased to the fatal dose there is a corresponding though feeble increase in thyroid activity In the comparable group of sensitized guinea pigs the slope of the dose response is steeper as the dose of toxoid is increased

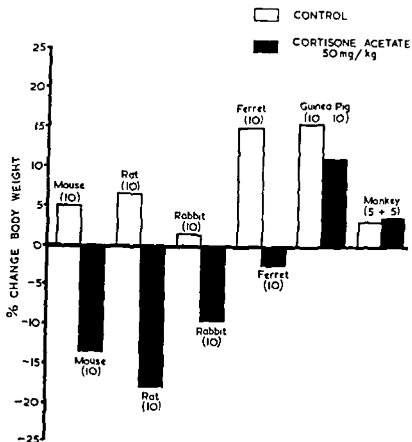


FIGURE 6 Effect of cortisone acetate on body weight in different species

TABLE I COMPARISON OF SPECIES

Species	Tuberculin Sensitivity	Ascorbic Acid Synthesis	Response to Cortisone
Man Monkey Guinea Pig	Readily induced	Ascorbic acid not synthesized	Resistant (antitoxin synthesis not depressed)
Rat Mouse Rabbit Ferret	Not readily induced	Ascorbic acid synthesized	Sensitive (antitoxin synthesis markedly depressed)

Antitoxin effect not known for Ferret

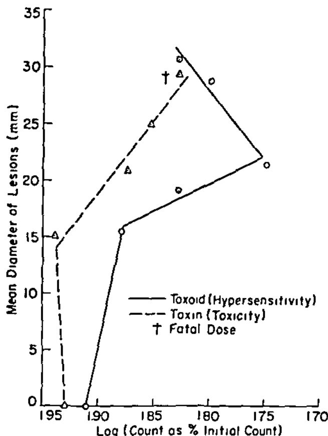


FIGURE 7 Effect of diphtheria toxin and toxoid on the release of I^{131} from the thyroid glands of guinea pigs

the thyroid stimulating effect increases more rapidly than in the toxin treated group until apparently overstimulation has less effect This type of allergic reaction has been termed total allergic damage to indicate that it is composed of immediate and delayed allergic reactions[1] The exact measurement and relationship of each type of allergic response to the activity of the thyroid gland provides one of our current research themes The above experiment shows that hypersensitivity to a bacterial antigen induces hyperthyroidism in guinea pigs more effectively than toxicity of that antigen Thus in this experiment the agent that damaged the tissues least stimulated the thyroid gland most In this case a vicious circle might be set up for hyperthyroidism increases the severity of both immediate[7] and delayed allergic responses in guinea pigs [8]

To conclude the confusion in the literature regarding the effect of the hormones of the adrenal cortex on immunity is resolved if the division

into cortisone-resistant and cortisone sensitive species is appreciated This division also provides a rational basis for the contrasting effects of bacterial stress on thyroid function in different species The two groups of species have other analogous metabolic features in common which profoundly affect their response to allergens (Table I) The potential significance of these observations has been outlined elsewhere [8]

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GENERAL DISCUSSION

JOSEPH H SHAFFER (Detroit Michigan) I am very glad to hear Dr Long's paper because for some time we have suspected that patients who have certain metabolic disorders will not respond very readily to treatment with antigenic extracts I am thinking mostly of the hypothyroid individual who requires thyroid therapy before we can see or expect much response to hyposensitization therapy

I suspect that the same situation applies in patients who have anemia In other words we must correct the deficiency before we can expect worth while results from hyposensitization with foreign protein substances

GEORGE L WALDBOTT (Detroit Michigan) In conjunction with the question of inheritance of allergy and of factors modifying allergenic responses three points should be stressed

The degree of exposure to an antigen appears to have a definite bearing on the development and the intensity of allergic manifestations For instance we frequently encounter a history such as My first attack of hay fever started while I was going through a field of ragweed or when I played in a haystack In other words the intensity of an antigenic invasion of the body is likely to be a factor in the development of allergy

Another significant factor is the frequency of exposure to the antigen Experience tells us that a patient who is exposed to an antigen frequently at specified time intervals is more likely to develop sensitivity

The time interval between such exposures has not been adequately investigated in clinical medicine For instance a patient exposed to penicillin at two week or three week intervals rather than daily is much more likely to develop sensitivity to penicillin

This brings up the question do humans acquire allergy? We know that contact dermatitis can be produced in most normal individuals without an inherited tendency to allergy My thoughts on this point became crystallized when I studied the mechanisms and the pathology of fatal human allergic shock some twenty years ago

I observed that there is one type of fatal shock which should rightly be termed human acquired anaphylaxis It is characterized by capillary permeability in the lungs by extravasation of blood cells and edema fluid into the pulmonary tissue by absence of eosinophilia by absence of reagins in the blood

This type of shock must be contrasted with what we see in a patient who is already allergic or who exhibits an inherited tendency to allergy

when he dies of a penicillin reaction There is bronchospasm emphysema eosinophilia increased secretion of mucus into the bronchi

Both the acquired and the inherited type of shock used to be most prevalent from injections of horse serum now they are most frequently encountered in fatalities from penicillin and salicylates

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CHAIRMAN CHASE Would you consider that what we need is more experimentation?

DR WALDBOTT I place much reliance upon the excellent experimental work which has been presented to us here respecting both animals and humans There can be no objection to doing careful experimental studies on humans However if we as clinicians keep our eyes and ears wide open when we face our patients I believe we can obtain much additional information on points brought to light in this excellent symposium to which the animal experiment cannot provide the complete answer

CHAIRMAN CHASE Since I do not hear another question from the audience I call upon Dr Shaffer to make our intended adjournment official I trust that all of you will have found elements of value from your attendance here during the symposium to be carried back to laboratory study, or office

DR SHAFFER It would be unfair to close this three day symposium without congratulating the audience for their presence the speakers for their participation and their splendid papers and our National Advisory Committee for their cooperation and assistance in formulating this three day program

On behalf of the Henry Ford Hospital Staff our thanks and very best wishes to all of you

XIII

Banquet Speech

Chairman GEOFFREY EDSALL, M.D. (*Washington, D.C.*)

48

Are We Too Trigger-happy?

TABLE TALK ABOUT SOME INITIATORS OF THE INFLAMMATORY RESPONSE

A ASHLEY MILES MD

(London England)

No one I am sure you will agree who has enjoyed the hospitality of the Henry Ford Hospital during these two days and especially at this banquet, should have any reason for being a prey to apprehensions But in so far as it s possible to be other than contented I confess to you I am both apprehensive and penitent I am penitent because of the question which in a moment of enthusiasm I gave to Dr Shaffer as a title for what I wanted to say tonight — Are We Too Trigger happy Those of you who believed that under a thin guise of rambling thoughts about allergy I was going to make a masterly analysis of the somewhat explosive subject of East West relationships have been sadly misled You are like the victims of the enterprising gentleman who finding himself with the stock of a respectable but bankrupt bookseller on his hands advertised in suitably chosen magazines his readiness to sell a copy of What Every Young Girl Ought to Know in plain wrappers and within a month had disposed of one hundred thousand copies of the Bible — you can t voice your complaints without convicting yourselves of impropriety But I get little comfort from this reflection I was angling for your attention having got it under false pretenses I now realize I must hold it for as long as my friend Geoff Edsall here can impose a chairman s authority on an outraged audience cheated of a funeral oration about civilization as we know it

There is some mitigation of your disappointment in that though my subject is not explosive it is certainly inflammatory because the triggers I am going to suggest we are too happy about are those which initiate the processes of inflammation In choosing to talk about inflammation I have no scruples about getting away from allergy as such At this stage of our symposium you will probably welcome a diversion even if it s a small one and it certainly is small because the step from the allergic to the phlogistic *is not* a big one The step from an allergen to a phlogistogen is sometimes even smaller indeed to a confirmed phlogisto

geneticist like myself (having got as far as phlogistogen I could not resist going one better but this new professional title is so horrible I lay claim to it for this occasion only) – to the confirmed phlogistogeneticist the animal which has become allergic is chiefly remarkable for having added one more substance to the enormous list of inflammatory stimuli to which it was already susceptible

The endothelium that separates the circulating blood from the tissues in the normal animal is nicely adjusted to maintain a highly selective exchange of materials between the two One common feature of nearly all allergic and inflammatory reactions is a dislocation of this endothelial barrier

The dislocation is revealed in many ways two of the most obvious because they are readily detected on a macroscopic scale, occur in the small blood vessels at a very early stage of inflammation The inner wall of the vessel becomes sticky and the wall itself becomes leaky that is to say there is an increase in permeability or if you prefer it, porosity to large molecules Moreover judging by the coincidence in the experimental animal of stickiness and leakiness in both time and place they are expressions of the same fundamental change in the vessel wall[12 17 23] If then we can find what it is that triggers off the increase in vascular permeability it is reasonable to believe that we shall have in our hands at least one of the threads needed to guide us in the labyrinth of the acute inflammatory reaction There s no scarcity of candidates for the leading role in the trigger mechanism They range from unidentified permeability factors released when tissues are pounded in a mortar to precisely identified substances like 5 hydroxytryptamine They include the products of proteolysis which in connection with anaphylaxis were proposed half a century ago [9] Sometimes the enzymes activated in this process and the native antienzymes whose removal is supposed to start it are identified simply as proteases and antiproteases sometimes they are identified with more precision as components, say, of the plasmin system [6] Of the active lytic products we have various polypeptides on the one hand and on the other hand histamine released enzymically from its hypothetical bondage to a protein Histamine of course is the other time honored candidate and as a trigger has a strong claim to stand in its own right quite apart from any proteolytic sequence

Now trigger happiness is a distemper to which anyone who seeks for a trigger mechanism in pathological situations may succumb It s a common ailment because pathological situations are so obscure that any ray of light is apt to be hailed as the dawn of a new day Let me illustrate the obscurity A simple principle and one surely fundamental in the search for the trigger is correlation in time One of the reasons I suspect why we understand so much about acute anaphylaxis is that being sudden

and short lived it demands of its investigators the demonstration of a close coincidence of cause and effect in time and place Delayed allergic reactions and inflammatory reactions in general are less demanding They invite investigation in the full bloom of their maturity and the initial trigger is often guessed at by extrapolating in time to what is imagined to have been the beginning Experimental pathologists (and in this respect I am typical) are devoted to the study of the more violent and unnatural kinds of inflammatory agent — such as doses of croton oil or pathogenic staphylococci that within one or two days will induce copious exudation into a serous cavity or an enormous purulent abscess in the subcutaneous tissues There s undoubtedly much to be learned from lesions of this severity and age but they are not I submit ideal guides to facts or hypotheses about trigger mechanisms To expect them to be so is rather like hoping to reconstruct the whole of *Hamlet* by listening to the dying speeches of the four characters whose corpses litter the stage in the final scene In the whole of this scene there s only one remark that gives us a decent clue to the cause of the trouble — Hamlet s damning the King as a murderous Dane — and how far will that go in explaining why the Queen Laertes and Hamlet himself have also been polished off

The last act of *Hamlet* came to mind as an illustration of my point because in the final scene half the actors like much of the tissues in the mature inflammatory reaction are dead But on rereading it not only did I find the illustration more apt than I had remembered but I was rewarded with a compelling discovery in Shakespeare scholarship — a discovery which at the risk of even greater irrelevance than I have so far indulged in professional pride impels me to share with you There were you may remember eight years in Shakespeare s early manhood about which history is wholly silent During this time he is variously supposed from the internal evidence of the plays to have been schoolmaster lawyer soldier sailor poacher call boy in the theater holder of horses for the playgoing gentry and even a confirmed alcoholic

Now listen to Horatio s masterly summary of the confused situation in *Hamlet*

> And let me speak to the yet unknowing world
> How these things came about so shall you hear
> Of carnal bloody and unnatural acts
> Of accidental judgments casual slaughters
> Of deaths put on by cunning and forc d cause
> And in this upshot purposes mistook
> Fall n on the inventors heads

Think of our bizarre injections into numberless experimental animals our hemorrhagic lesions and above all the vicissitudes of our contradictory hypotheses and what doubt can remain in your minds that for

some of those lost years the sardonic young Shakespeare was apprenticed to an experimental pathologist?

Ideally the time to seek for the trigger is during the transient little inflammatory response induced by waggling a discreet microneedle in the vicinity of a capillary loop We shan't go far wrong if we work on a bigger scale but we must stick to minimal stimuli like a subnecrotizing dose of a toxin[6] or a short exposure to ultraviolet light and we usually find in these conditions that the significant increases take place within an hour or two and before the grosser manifestations of inflammation[1, 11]

What kind of trigger substance are we looking for? First it must not be present in effective amounts in normal tissue Second bearing in mind the tiny stimuli that can set it off it must be readily activated on the spot or readily released from somewhere else Third since the reaction in its simplest form is transient we might expect also to find substances that will inactivate the trigger substance

My own heart searchings about trigger mechanisms began some six years ago with an attempt to explain why blood capillaries that had already responded to a permeability factor had thereby been made resistant to permeability factors in general I wanted to fill the extravascular tissues of the guinea pig's skin with a solution of high osmotic pressure and in the simple minded belief that it would serve as a solution of bland autologous proteins I used the animal's own serum for the intracutaneous injection It didn't do what I hoped of it but it did induce resistance to permeability factors and this without itself first increasing the vascular permeability Next succumbing to the ingrained habits of twenty years of immunology I tried to titrate the resistance inducing substance by injecting serial dilutions of the serum and found instead that a powerful permeability factor had been unmasked[13]

The striking feature of the phenomenon was the easy and rapid activation of the factor from an inert profactor In this respect at least the profactor qualified for a role in a natural trigger mechanism for it seemed likely that what occurred in the test tube as a result of simple dilution might be achieved in the body by equally gentle means

My colleagues and I located the profactor among the alpha globulins of the guinea pig plasma and found there also an inhibitor of the factor capable of slowly neutralizing the activated profactor The factor itself even in our relatively impure preparations had about the same permeability increasing potency as histamine[3] Our next step and one which proved to be quite a labor was to ensure that this system — of activable profactor plus native inhibitor — was not peculiar to the guinea pig

Comparative studies whether anatomical or physiological have one notable virtue — as a colleague of mine once remarked you're bound to get somewhere If you have found something in one species and then find

the same thing in another species that's very interesting and if you fail to find it that's very odd Our findings were interesting in general and odd only in small particulars that I needn't go into The profactor-inhibitor system is present in the plasma of man rabbit rat and guinea pig the profactor itself being among the alpha and beta globulins and the inhibitor among the albumins and alpha globulins The profactor is plentiful each milliliter of plasma in the three laboratory animals contains activable protein which in its capacity to increase permeability is equivalent to over 300 µg of histamine Moreover the factors in each species are active in heterologous animals and are in all cases antagonized by the same inhibitors including the native inhibitor of the plasma [23, 16, 1, 6] This together with some reasonably good evidence that a similar profactor is present in cat dog horse ox and mouse suggests that the profactor inhibitor system is a regular feature of at least mammalian plasma

Our right by the way to talk of a profactor inhibitor *system* rests entirely on the behavior of serum in the test tube where rapid activation is followed by slow inhibition we have as yet no good evidence that the system behaves as such in the body But incomplete as our researches are it would be inhumanly perfect especially after dinner to refrain from seeing how these permeability globulins of the plasma fitted into current notions about inflammation

The factors are presumably enzymic because their activity on a molar basis is several hundred times that of histamine they are presumably proteases of some sort because their activity is irreversibly destroyed by soya bean trypsin inhibitor [23] and di *iso*propyl fluorophosphonate [1] and if proteases they presumably have a restricted range of action since we still lack any firm *in vitro* indication of a substrate

They would thus fit readily into a proteolytic trigger mechanism That is hardly surprising because such mechanisms being largely hypothetical are very accommodating The more detailed and therefore less accommodating proteolytic schemes in which histamine is included as an end product are ruled out because we can find no evidence that the serum factors either release histamine or are specifically inhibited by the antihistaminic drugs So the two systems supposed proteases and their consequences on the one hand and histamine and its antecedents on the other are either rivals for the role of trigger or share the honor between them one or the other being trigger according to circumstance It will sharpen the contrast between the two to regard them as rivals and since it would be idle to pretend that I have no axe to grind I propose before I deal with the serum factors to see first what can be done to eliminate histamine

Let me at the outset handsomely admit that the histamine scheme is highly plausible Bound or segregated histamine is plentiful common to

many tissues and to many animals It has one established physiological role — that of a gastric hormone,[3] and it is a potent and successful mimic of some of the features of inflammation That it doesn t imitate them *all* is nothing against it, we are looking for a trigger, not something that is trigger, cartridge, and target rolled into one Above all, it is fairly easily released from its bound or segregated state Against this we may say that except in a few pathological conditions (which I hasten to concede to this audience include some of the more striking allergic states), histamine plays no demonstrable part in the regulation of blood flow[2] or vascular permeability Moreover its wide distribution among animals and plants might be evidence not of its general functional importance but merely that living tissues having accepted histidine as a building block for protein share the problem of disposing of the inevitable consequences of having decarboxylases as well as part of their enzymic machinery Another argument against histamine as a mediator of vascular reactions is that the tissues may have ideas about recognizing and using histamine which are very different from those of the pharmacologist, as an obviously reactive amine histamine may have more immediately useful but as yet unrecognized chemical functions in the cell Let me go further and suggest that this extragastric histamine has no function at all Some of it is absorbed from the gut and some presumably formed in the body from histidine Is it not possible that much of the histamine we find in the tissue is on the way out that like urea or bile pigments though not quite like them in being excretory end products it is at a penultimate stage of excretion?

However the story of histamine as nothing but a metabolite is unconvincing because certain tissues take the trouble either to bind it or to tuck it away inside cell granules This fact is disturbing enough for the antihistaminist, and more so when we find an abundance of cells bearing such granules in the vicinity of blood vessels[18] The fact of a paravascular distribution of mast cells is less awkward for my argument than the fact of storage Proximity to blood vessels after all is a situation that most tissues find hard to avoid, and mast cells may well be near vessels for their own benefit rather than as servants of a vascular response The storage, of course doesn t necessarily imply storage for release If histamine with its unpleasant vascular potentialities *has* to be present in the tissues it would clearly have to be segregated from the vessels in some way much as trypsin has to be segregated from the tissues of the organ that synthesizes it If histamine is indeed stored in readiness for a vascular response to injury, the tidy evolutionist might at first sight demand that the mast cells should have been a little closer to the vessels On second thoughts he would realize that histamine is sufficiently diffusible to offset that disadvantage and he might plausibly argue that some remoteness of

the mast cell from blood vessels is a positive advantage because injury to the outlying mast cell would release a hormonal flow of histamine sufficient to induce protective responses in distant capillaries My argument appears to be coming down on the side of histamine so I hasten to redress the balance by recalling the few natural or experimental pathological states in which the antihistaminic drugs seem to have any specific effect When they fail to antagonize effects we might expect are due to histamine they may do so because the histamine is released intrinsically that is in cells which respond to the histamine they themselves release[4] But their failure to influence inflammation in general cannot be excused in this way because unless we postulate the presence of bound histamine in the vascular endothelium itself — for which I know of no evidence — we must regard vascular reactions as mediated by extrinsic histamine

And even on those occasions when the drugs are inhibitory their value in identifying a histamine effect is suspect especially when they act only in large doses There was once a farmer at a county fair who was offered a magnificent but solemn looking horse at a very low price The seller was quite frank about getting rid of the animal the horse was a good one but was no use to him You see he said I grow grapefruit and he sits on grapefruit

Sits on grapefruit said the farmer

Yes sits on grapefruit

Well said the farmer I raise cattle so I reckon that won t worry me bought the horse and rode it home On the way he had to ford a river and when he was halfway across the horse stopped suddenly and slowly sank back on its haunches Nothing would budge it and at last in despair the farmer left it sitting immovably in midstream with an obstinate look on its face He waded ashore and went back to complain bitterly about his bargain Aw heck said the fruit farmer I clean forgot to tell you he sits on fish too

The moral of this venerable shaggy dog story is Nothing is ever as specific as it s first said to be and that goes for inhibitors as well as for habits

The instances of less convincing inhibition of natural phenomena by the antihistaminics are few but the difficulties of proving their specificity are many I shall not try to discuss them, but will imitate the preacher who said of a controversial text My friends we have come to a passage that has been a stumbling block to many of us Let us confront the difficulty and look it boldly in the face And now having looked it boldly in the face let us pass on

Let us pass on to a less obvious but real obstacle in the progressive demotion of histamine In a recent symposium on neurohumoral transmission one speaker[8] posed the question Dare we conclude from the

presence of a large amount of substance that it s of particular importance I found it surprising that a question like this should be asked at all because most discussions about the significance of an endogenous substance seem to me to be based on an entirely opposite view From this opposite view point the bald answer to the question Why do you think histamine has something to do with the trigger mechanism is Because it s there which amounts to saying that all the features of the living organism have a purpose otherwise they would *not* be there In the nineteenth century this frankly teleological belief in the consistently purposive behavior of living tissue would have been both acceptable and respectable Nowadays it s less respectable partly because it s so vulnerable to argument and partly because biology manages to get on nicely with fewer transcendental principles

It s vulnerable because no one is really in a position to say what the ultimate biological purpose is it s therefore difficult to refute anyone who thinks of an equally transcendental purpose with opposite intentions I would not suspect that devout mid Victorian naturalist Philip Gosse, of devising arguments which were intended to pose a metaphysical dilemma but he provided an admirable illustration of the kind of unanswerable refutation I have in mind when he was confronted with the inadmissible but nevertheless indubitable evidence of the fossil sequences in sedimentary rocks They were put there he thought to test the faith of those tempted to believe in evolution

If there is no need for me to suggest that histamine has been put in the mast cell without any discoverable function to test the faith of those tempted to disbelieve in teleology it is because there are more pertinent ways of regarding its usefulness We needn t guess at the purpose of this or that feature but instead can ask what advantage it gives to the individual or the species in the struggle for existence The replacement of purpose by the concept of survival value doesn t make the job of the biologist easier but though answers in terms of survival value are hard to come by once obtained they are much less misleading than the answer given by the teleologist

The change is not all gain I fear There s more logic in it for us but the newer biological world has become much less poetical much less comforting much more drab Our more metaphysical forefathers in biology could aptly misquote at us lines from Clerk Maxwell s ode to Hermann Stoffkraft, Ph D

> But when thy Science lifts her pinions
> In Speculation s wild dominions
> We treasure every dictum thou emittest
> While down the stream of evolution
> You drift expecting no solution
> But that of the survival of the fittest

Even the biologists who find teleology most uncongenial either as an attitude or a scientific technique and resolutely paddle their empiricist canoes down the stream of evolution must admit its attractions if only because it can reassure them — as we all need reassuring — that when they are on to what they feel is a good thing it is likely to prove immensely significant.

There are of course robustly empirical ways of saying It must be significant because it s there but they may lead to teleology by a back door route Suppose we roundly state that most of the histamine stored in the body is useless conceding its possible usefulness in some living organisms but relegating it in mammals to a mere evolutionary rest an ancient ingrained once useful biochemical mechanism which mammalian tissue is burdened with as the price of enjoying the metabolic powers it needs for the purely local manufacture of gastric histamine The nonteleological answer to *this* argument is that the tissues are clearly expending a small but appreciable amount of energy on keeping up this useless activity energy that would otherwise be devoted to survival and if it is in fact an overall disadvantage to waste energy in this way it is probable according to the genetical theory of natural selection that in Mendelian populations like those of the higher animals we are considering unnecessary histamine production would have disappeared in a much shorter time than these animals have been flourishing on earth The translation of this hypothetical answer into a real one depends however on knowing how much of a disadvantage it is and that we do not know If you can compare survival rates you can estimate disadvantages and presumably if you can measure disadvantages you can predict survival rates but you can t assign a measure of disadvantage to a feature just by taking thought If therefore you assert of a well established and stable feature thought to be merely useless that if it were useless it would be disadvantageous you are saying in effect that only useful features persist And when you have made persistence a criterion of usefulness you are indeed back on the metaphysical bandwagon and differ from the out and out teleologist for whom all activity is purposeful only in generously giving the evolutionary process a bit of elbow room allowing it to make mistakes but only on the condition that they are corrected within a few score thousand years

But even conceding that useless features must ultimately disappear with the example of the hundred odd million years during which the great Mesozoic reptiles managed to flourish in spite of their egregious bulk and ornamentation who are we upstart mammals to cry down a bit of histamine overproduction as a handicap in the struggle for existence?

I can t hope to have convinced you with this piecemeal exclusion of histamine from the inflammatory scene indeed I haven t convinced myself My aim rather was to show that though we rely on plausibility

in the absence of proof, our ideas about the plausibility of a biological explanation are not always as rigorously scientific as they might be The lines of my inquiry are valid enough but as weapons for leaving the scene clear for a rival trigger mechanism they are two edged because most of them are equally forceful when applied to the plausibility of *any* endogenous trigger substance If you are proved too trigger happy about histamine I am by the same tokens certainly too trigger happy about the *serum factors Since neither of them has been proved unequivocally to* be a trigger the decision between the two at present depends on a comparison of plausibilities It remains to examine some secondary evidence and to ask which of the two systems is the better placed functionally and topographically as a trigger mechanism

There s quite a lot of histamine about in the tissues and some of it in close relation to vessels In a contest with most of the other endogenous substances that increase permeability histamine wins easily because *like that dashing cavalryman General Nathan Forrest it gits thar fustest* with the mostest The profactor however is there all the time not only inside the blood vessel but in the tissue fluids as well [14] Like other serum proteins both the profactor and its inhibitor are continually passing in small quantities through the tissues to rejoin the blood stream via the lymphatics and at least as far as the guinea pig s skin is concerned the intercellular concentration of profactor is well in excess of the amount needed to increase the permeability of all the skin capillaries Moreover since we have found no *acute* pathological state in which the plasma demonstrably loses its profactor [4] the tissues can depend on a copious supply

As regards ease of activation histamine is released from tissue cells by a wide variety of substances such as amines enzymes bacterial toxins surface active agents antigen antibody reaction Many of these agents are highly reactive substances in contrast the *in vitro* activation of the serum profactor seems gentle Dilution itself is not the whole story because activation is very slow when serum is diluted in Polythene tubes [1] It is very rapid when serum is exposed to a large glass surface by gentle shaking with ballotini [11] or when serum is exposed to agar starch granules *or powdered cellulose The dilution effect we first observed in glass tubes* seems in part to be due to the decreased concentration of profactor relative not to the diluting fluid but to the available glass surface

The essence of the process seems to be exposure of profactor to an alien wettable surface I think this is a comparatively gentle procedure but living among colleagues who regard even a little frothing in a protein solution as the beginning of a Gadarene stampede towards denaturation I m quite prepared to believe that the surface activation of a protein profactor in this way is quite as brutal in a physicochemical sense as any

of the reactions that release histamine Nevertheless the notion that a tissue cell normally bathed in a fluid containing profactor has only to be injured to the point where its surface properties change in order to activate that profactor is to my mind a highly plausible model of the kind of trigger mechanism we are looking for and the slowly acting native inhibitor of the factor which is also present in the tissue fluid is at least as plausible a restorer of the normal state as the oxidases and so forth which draw the teeth of histamine

There s no difficulty in showing the activation of the globulin factor in some pathological states [20] — or that it is present as profactor in mature exudates Indeed since mature exudates contain a substantial amount of all the plasma proteins it would be surprising if profactor were absent But for demonstrating activation on a small scale in early lesions we shall probably have to rely on inhibitors with all their limitations of specificity We hope to avoid the limitation of pharmacological inaccessibility to inhibitors because there can be little doubt that the profactor is extrinsic in Dale s sense of the word

Histamine is presumed to act directly on the capillaries the presumption I suppose is based partly on the smallness of its molecule and partly on the fact that it acts quickly The globulin factor acts just as quickly on the capillary plexus [3] and I see no reason why it should not act directly If the factor is a protease the most plausible small molecular intermediate between it and the capillary wall would be a polypeptide for polypeptides are formed quite rapidly in physiological conditions [9] and some polypeptides are permeability factors However the protease might in nature have a more restricted action (like that of thrombin on fibrinogen) which resulted not in general proteolysis but let us say in a depolymerization of the endothelial cement substance If increase in capillary permeability depends on broadly intercellular events the need for a mobile small molecular mediator is less evident than it is for example in the cellular sites where acetylcholine has to act The economy of the sharply localized inflammation may in fact be better served by a large molecular mediator for in such circumstances a highly mobile little molecule might be a source of widespread embarrassment

Here Mr Chairman my competition in plausibilities comes to an end and with it my ascent into rarefied air of the higher criticism Like the early fire balloonists I have come down to earth because my hot air is exhausted Between the two trigger mechanisms I have so arbitrarily chosen to illustrate my theme the honors I think are easy and I leave it to you to decide about trigger happiness I cannot claim that my scrutiny of the opening scenes of the action has got us much further in understanding this *Hamlet* like puzzle of inflammation Indeed you may feel that by cold shouldering histamine I have deprived *Hamlet* of its Prince

of Denmark if you do I offer the excuse that Hamlet himself like histamine in the inflammatory drama appears so often to be promising more than he actually accomplishes

I have ignored many of the bit players like 5 hydroxytryptamine and the various polypeptides, which have been allowed little more than an appearance on the playbills This has been an essay in the principles of dramatic criticism rather than an analysis of the drama and here I have certainly failed to apply some important critical principles To take one instance I have assumed without any real justification that there is a basic inflammatory pattern common to all animal tissues, to take another I have not considered whether those twin events vascular stickiness and leakiness occur in all inflammatory responses Happily for me, it s too late to go into these things now

It is to be expected of a critic no matter how olympian his detachment no matter how open minded his appreciations that he will play up one actor at the expense of another In dismissing Hamlet I may perhaps have overemphasized the King as the villain of the piece I have done so I plead in extenuation with all the hesitations of the ill rehearsed actor of whom it was said that he played the King as if at any moment he expected someone would play the Ace Besides being tentative I have tried as well to be a little more than or perhaps a little less than open minded Merely having an open mind as Chesterton said is nothing the object of opening the mind as of opening the mouth is to shut it again on something solid If in my anxiety to say something solid I have like the capillary been stimulated to undue leakiness I trust you haven t found the going intolerably sticky

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